Adenosine in health and disease

a human *in vivo* study on genetic, metabolic, and pharmacological determinants of the cardiovascular effects of adenosine

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ISBN: 978-90-9021625-6

Cover: Gerri van den Broek & Niels Riksen
Printed by: PrintPartners Ipskamp B.V. Enschede

The research presented in this thesis was supported by a grant from the Netherlands Organization for Health Research and Development (ZonMw).

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Een wetenschappelijke proeve op het gebied van de
Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de Rector Magnificus prof. dr. C.W.P.M. Blom,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op woensdag 11 april 2007
om 15.30 uur precies

door

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geboren op 3 juli 1978
te Arnhem

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LIST OF ABBREVIATIONS

ADA	Adenosine deaminase
Ado	Adenosine
AdoHcy	Adenosylhomocysteine
AdoMet	Adenosylmethionine
ADORA2A	Adenosine A_{2A} receptor gene
ADP	Adenosine diphosphate
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AK	Adenosine kinase
AMP	Adenosine monophosphate
AMPD	Adenosine monophosphate deaminase
AMPD1	Adenosine monophosphate deaminase-1 gene
ATP	Adenosine triphosphate
CBS	Cystathionine beta-synthase
DBP	Diastolic blood pressure
ENT	Equilibrative nucleoside transporter
FBF	Forearm blood flow
FFA	Free fatty acids
FVR	Forearm vascular resistance
Hcy	Homocysteine
HPLC	High-performance liquid chromatography
HR	Heart rate
Ino	Inosine
IP	Ischemic preconditioning
Isch Ex	Ischemic exercise
MAP	Mean arterial pressure
MAT	Methionine adenosyl transferase
Met	Methionine
MTHFR	Methylenetetrahydrofolate reductase
MTX	Methotrexate
5′-NT	5′-nucleotidase
SBP	Systolic blood pressure
SNP	Single nucleotide polymorphism
UV	Ultraviolet

CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

BACKGROUND

Already in 1929 it was recognized that adenosine, the nucleoside base of the high energy molecule adenosine triphosphate (ATP), can serve as an extracellular signaling molecule, which reduces heart rate, lowers blood pressure, and increases coronary flow in anesthetized animals (1). In the early 1960's Berne and Gerlach *et al* proposed that during hypoxia, myocardial cells release adenosine, which subsequently mediates the local metabolic control of coronary blood flow (2, 3). Since the publication of these seminal papers, many studies have addressed the regulation of the extracellular adenosine concentration in normoxia and hypoxia, and the functions of adenosine in cardiovascular (patho)physiology (4, 5). By stimulation of membrane-bound G-protein coupled receptors, adenosine has negative inotropic, dromotropic, and chronotropic effects on the heart, induces vasodilation in most vascular beds, modulates activity of the sympathetic nervous system, and enhances tolerance to ischemia (6-8). Also, adenosine receptor stimulation inhibits thrombocyte aggregation and inflammation and modulates vascular cell proliferation and death (9-11). Together, these effects have the potential to reduce tissue injury in situations of impaired oxygen delivery, which is illustrated by the observation that adenosine receptor stimulation during ischemia and reperfusion reduces myocardial infarct size (12).

In situations of impending tissue danger, such as ischemia or hypoxia, changes in the metabolism of adenosine result in a rapid increase of the extracellular adenosine concentration (13-15). In an autoregulatory way, subsequent adenosine receptor stimulation increases ischemic tolerance of the affected tissue and reduces local tissue injury. In 1984 Newby introduced the term "retaliatory metabolite" to describe these immediate local protective effects of adenosine (16). More recent studies show that, in addition to these immediate effects, adenosine receptor stimulation also has the potential to inhibit the development of atherosclerotic plaques and promotes angiogenesis, which will ensure tissue integrity in the long run (17-20).

Bearing in mind the various beneficial cardiovascular effects of adenosine, it is logical to assume that changes in the metabolism of adenosine could affect individual cardiovascular risk. Indeed, a common variant in the gene encoding for one of the isoforms of the enzyme adenosine monophosphate deaminase (*AMPD1*), which predicts augmented adenosine formation during ischemia, is associated with reduced cardiovascular mortality in patients with coronary artery disease (21). As such, all factors, either genetic, metabolic, or pharmacological, that modulate the kinetics or dynamics of endogenous adenosine could potentially affect individual cardiovascular risk.

In this thesis, the results of a series of human *in vivo* studies are described, which are focused on two important effects of adenosine: vasodilation and protection against ischemia-reperfusion injury. We aimed to identify genetic, metabolic, and pharmacological determinants of the cardiovascular effects of adenosine in humans *in vivo.* In order to understand these studies, and the theoretical concepts on which these studies are based, we will give an overview of the metabolism of adenosine, the effects of hypoxia on adenosine metabolism, and the cardiovascular effects of adenosine receptor stimulation.

REGULATION OF THE INTERSTITIAL ADENOSINE CONCENTRATION

The regulation of extracellular adenosine is critical for the interaction of adenosine with its receptors and the subsequent effects. The extracellular adenosine concentration is determined by several proteins involved in formation, degradation, and transmembranous transport of adenosine (figure 1), which has been described in detail in excellent previous reviews (4, 22).

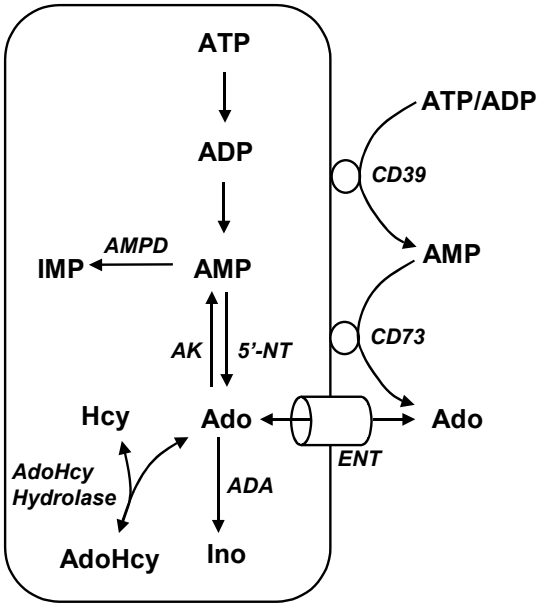

Figure 1: Schematic representation of the metabolism of adenosine. The abbreviations are explained in the list of abbreviations on page 9 of this thesis.

The intracellular formation is mediated either by an intracellular 5'-nucleotidase, which dephosphorylates AMP, or by the hydrolysis of S-adenosylhomocysteine (AdoHcy) by the enzyme S-adenosylhomocysteine hydrolase. Although the equilibrium constant of S-adenosylhomocysteine hydrolase favors synthesis of adenosylhomocysteine from homocysteine and adenosine, the reaction is driven in the opposite direction because both reaction products are rapidly removed from the cytosol (23). The transmethylation pathway provides a constant source of adenosine, although quantitatively less important than AMP hydrolysis, independent of tissue oxygenation, whereas during hypoxia AMP breakdown greatly increases and predominates the other pathway (22). An alternative pathway of AMP degradation is provided by the cytosolic enzyme AMP deaminase (AMPD), which catalyzes the irreversible deamination of AMP to inosine monophosphate (IMP) and ammonia. In humans, four AMPD isoforms have been described, and were named after the source from which they were initially purified: M (muscle), L (liver), E1 and E2 (erythrocyte), encoded by the different genes *AMPD1*, *AMPD2* and *AMPD3* (24). Although *AMPD1* is predominantly expressed in skeletal muscle, it is also expressed in cardiac muscle and aorta (25). The heart also contains the isoenzymes E1, E2, and L (26). Which pathway of AMP

catabolism predominates, depends on the species, cell type, and on the cellular metabolic state (4, 27). It is suggested that in cardiomyocytes, under well-oxygenated conditions, AMP is mainly converted to IMP, whereas in situations of increasing ATP degradation, conversion to adenosine may gain more importance (22, 28-30). In skeletal muscle, however, there is an almost stoichiometric increase in IMP during accelerated ATP degradation (31).

Extracellular dephosphorylation of AMP by membrane-bound ecto-5'-nucleotidase (CD73) is the final step in the enzymatic chain that catalyzes the breakdown of extracellular adenine nucleotides, and provides an alternative important source of adenosine (22, 32). Extracellular adenine nucleotides originate from various sources: ATP is released by exocytosis from sympathetic nerve terminals where it is co-released with norepinephrine (33), it is released by aggregating thrombocytes (34), and under hypoxic conditions ATP is released from various celltypes, such as erythrocytes and endothelial cells (35, 36).

Degradation of adenosine is mainly confined to the cytosolic compartment, where it is rephosphorylated to AMP by adenosine kinase, or deaminated to inosine by adenosine deaminase (22, 37). Based on the K_m values of these enzymes (approximately 0.5-2.5 μM for adenosine kinase, and approximately 30-100 μM for adenosine deaminase (38-40)), it is to be expected that rephosphorylation to AMP is the predominant pathway of adenosine metabolism in the normoxic situation, whereas during impaired oxygen delivery, when the adenosine concentration increases, adenosine deamination also gains importance. Indeed, approximately 90% of myocardial adenosine produced under normoxic conditions is salvaged by rephosphorylation (41). Under conditions of hypoxia, however, inhibition of adenosine deaminase results in a greater increase in adenosine concentration than inhibition of adenosine kinase (42).

As adenosine is a relatively hydrophilic molecule, transport of adenosine across the cellular membrane is facilitated by a concentration-gradient-driven equilibrative nucleoside transporter (ENT) (43). In some specialized tissues (e.g. intestinal and renal epithelial cells) there are also sodium-dependent concentrative nucleoside transporters, but they play only a minor role in the metabolism of adenosine in the heart and blood vessels (44). The equilibrative nucleoside transporters can be subdivided into two types based on the sensitivity to inhibition by nitrobenzylthioinosine (NBMPR) (43). Both types from humans have been cloned and are named hENT1 (NBMPR-sensitive) (45), and hENT2 (NBMPR-insensitive) (46). hENT1 is almost ubiquitously distributed in human tissues, transports a wide range of purines and pyrimidines (K_m for adenosine approximately 50 μM) and is highly sensitive to inhibition by dipyridamole. hENT2 is also expressed in a wide range of tissues, but it has a lower affinity for adenosine, and is less susceptible to inhibition by dipyridamole (43, 47). For an adequate interpretation of studies in rats, it is important to emphasize that the rat ENT1 is essentially insensitive to inhibition by dipyridamole (43). More recently, two additional ENT transporters have been characterized in humans. The hENT3, although present in cardiac tissue, has been demonstrated to be a lysosomal transporter and, therefore, is unlikely to contribute directly to regulation of interstitial adenosine concentrations (48). Adenosine transport by the hENT4, which is also present in the heart, is highly

pH-dependent, optimal at acidic pH, and could therefore be particularly important in the regulation of interstitial adenosine during ischemia (49).

As both intracellular and extracellular AMP hydrolysis contribute to adenosine formation, whereas degradation of adenosine is confined to the intracellular compartment, there is a continuous adenosine concentration gradient across the cellular membrane under normoxic conditions from extracellular to intracellular (50). Under well-oxygenated conditions, the cytosol thus represents a sink rather than a source of adenosine. As a consequence, blockade of the hENT1, e.g. by dipyridamole, will increase the interstitial/extracellular adenosine concentration (50).

THE ENDOGENOUS ADENOSINE CONCENTRATION

The extracellular concentration of endogenous adenosine under well-oxygenated conditions is determined by the sum of the processes of formation, transport, and degradation, as outlined above. In the last decades, many studies have been devoted to the measurement or calculation of the endogenous adenosine concentration in blood and (myocardial) interstitium. This has proven to be extremely difficult, as the half life of adenosine in blood is approximately one second, because adenosine is rapidly taken up and metabolized by erythrocytes (51). Moreover, concentrations in blood do not reflect interstitial concentrations because the endothelium acts as an active metabolic barrier for adenosine (52, 53). Several methods have been developed and used to estimate endogenous adenosine concentrations: determination of the adenosine concentration in coronary venous effluent of isolated animal hearts by HPLC (38); determination of adenosine in plasma, after the immediate addition of a "blocker solution", which contains inhibitors of the enzymes and transporters involved in adenosine metabolism (54); microdialysis techniques have been used to estimate (myocardial) interstitial adenosine concentrations (see "*Techniques to determine the concentrations and effects of adenosine in humans in vivo*") (14); and mathematical model analyses have been used to estimate cell surface concentrations of adenosine (50). The estimations of extracellular adenosine concentration during normoxia vary from 7-1000 nM (14, 55).

EFFECT OF HYPOXIA ON THE METABOLISM OF ADENOSINE

It was suggested already in the 1960's that the extracellular adenosine concentration increases during hypoxia or ischemia (2), but an integrated view on the mechanism of this increase has emerged only recently (15). Extracellular levels of adenosine increase profoundly after the onset of ischemia (13, 56). Also in humans *in vivo*, it has been demonstrated that skeletal muscle interstitial or intravascular adenosine concentrations are elevated during ischemia or hypoxia (51, 57, 58). Apart from increasing intracellular AMP availability due to accelerated breakdown of ATP, and from increasing ATP release from several cell types, such as erythrocytes and endothelial cells (15, 35), hypoxia has several effects on the expression and function of proteins involved in the metabolism of adenosine, which increase the extracellular adenosine concentration (15, 59). Hypoxia upregulates CD73 (ecto-5'-nucleotidase) and CD39 (ecto-nucleoside 5'-triphosphate diphosphohydrolase), which will increase extracellular adenosine formation (60, 61). Also, it was reported that prolonged hypoxia (24-48 hours) upregulates expression of cytosolic 5'-nucleotidase (59). Concomitantly, the expression of adenosine kinase decreases during hypoxia (59). In addition, hypoxia also induces an immediate inhibition of adenosine kinase. Because of the normal high

turnover of the AMP-adenosine-AMP metabolic cycle, this hypoxia-induced inhibition of adenosine kinase causes a major rise in adenosine concentration (42). Finally, hypoxia downregulates hENT1 expression in endothelial and epithelial cells (62, 63). It is still controversial whether these changes that occur during hypoxia result in a reversal of the transmembranous adenosine concentration gradient, which will induce adenosine transport from inside the cell outwards, or that the concentration gradient is merely decreased, which will reduce cellular uptake of extracellular adenosine. Some studies demonstrate that blockade of the ENT transporter attenuates the hypoxia-induced increase in extracellular adenosine (40), whereas most studies show that nucleoside transport inhibition during hypoxia/ischemia results in a further increase in extracellular adenosine (13, 51, 54, 64).

CARDIOVASCULAR EFFECTS OF ADENOSINE

The effects of adenosine are induced by stimulation of 4 widely-expressed membrane-bound G-protein coupled adenosine receptors (4, 6, 65, 66). These 4 receptors are cloned, and are named adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors. The A_1 and A_3 receptor preferably interact with the $G_{i/o}$ family of G proteins, whereas the A_{2A} and A_{2B} receptor interact with G_s proteins (6). These receptor subtypes vary considerably in their affinity for the endogenous ligand adenosine: the A_1 and A_{2A} receptors are high-affinity receptors, which are activated already by basal levels of adenosine, whereas the A_{2B} and A_3 receptor have lower affinity profiles (6). By activation of these receptors, adenosine induces a wide variety of effects in virtually every organ system (5, 10, 65, 67). In this section, the description of the effects of adenosine will be limited to those effects that could potentially contribute to the cardiovascular protective properties of adenosine, and which are relevant to the different experiments presented in this thesis. Several previous reviews are focused on the effects of adenosine on the kidney (68), the brain (69), the lung (70), and on the immune system (10, 71).

By acting on A_1 receptors in the sinus node, the atrioventricular node, and cardiomyocytes, adenosine has negative chronotropic, dromotropic, and inotropic effects (7). Adenosine is a potent vasodilator in most vascular beds by acting on A_{2A} receptors, and in some tissues A_{2B} receptor stimulation also induces vasodilation (65). Previous studies have demonstrated that adenosine receptor stimulation is involved in post-ischemic reactive hyperemia in the forearm (57, 72). In contrast, adenosine acts as a vasoconstrictor in renal arteries (73). Although an intact endothelial lining is not obligatory for the vasodilator response to adenosine in *in vitro* studies (74), there are several lines of evidence that the vasodilator response to intraluminally applied adenosine is endothelium-dependent. Firstly, several studies have shown that intravascular adenosine does not pass the endothelial lining, which acts as a strong metabolic barrier to adenosine (52, 53). Secondly, infusion of adenosine linked to macromolecules, unable to reach vascular smooth muscle cells still produces vasodilation (75). Finally, human *in vivo* studies have shown that the vasodilator effect of adenosine in the forearm can be inhibited by a nitric oxide synthase inhibitor (76), although this could not be confirmed in another study (77).

Adenosine receptor stimulation is involved in modulation of the activity of the sympathetic nervous system and autonomic cardiovascular control on different levels (67). Firstly, adenosine

produces a widespread activation of afferent fibers from various organs, including the heart (78), forearm (79), and kidney (80). In addition, adenosine potently stimulates carotic chemoreceptors, which explains most of the cardiovascular effects of intravenous adenosine in conscious humans (81). Adenosine increases blood pressure and stimulates ventilation when injected into the aorta proximal to the chemoreceptors, but decreases blood pressure (by direct vasodilation) when injected into the descending aorta (81). Indeed, systemic administration of adenosine increases blood pressure, heart rate, and muscle sympathetic nerve activity (MSNA) in healthy controls, whereas blood pressure is decreased and MSNA is unaffected in patients after bilateral carotid body tumor resection (82). In addition to its effect on afferents, adenosine has also been shown to inhibit neurotransmitter release from sympathetic nerve terminals by activation of presynaptic A_1 receptors (67, 83). Also in human *in vivo*, endogenous adenosine inhibits norepinephrine release from sympathetic nerve terminals in the forearm during sympathetic activation (84, 85).

By stimulation of A_1 and A_3 receptors on cardiomyocytes, adenosine is one of the main triggers of ischemic preconditioning: the phenomenon that brief episodes of ischemia render the myocardium more resistant to a subsequent more prolonged ischemic insult (86, 87). A more extensive introduction of the role of adenosine in preconditioning is found in chapters 4.1 and 4.2 of this thesis.

By activation of A_{2A} receptors, adenosine inhibits thrombocyte aggregation (88, 89). Moreover, adenosine inhibits thrombin-induced expression of P-selectin on platelets via the A_2 receptor (90). In the early 1980's it was discovered that adenosine receptor stimulation on inflammatory cells induces potent anti-inflammatory effects (91), and since then many studies were aimed to further elucidate these effects (10, 71, 92, 93). Considering the anti-inflammatory effects of adenosine, a similar paradigm has arisen as was previously proposed for the metabolic regulation of coronary blood flow: during excessive inflammation, the concentration of adenosine rises, which subsequently downregulates activity of immune cells by activation of A_{2A} receptors (10, 71). There is an emerging view that many of the protective actions of adenosine in reperfused tissues may stem from modification of inflammatory responses (8). In animal studies of cardiac ischemia, administration of specific A_{2A} receptor agonists at reperfusion reduces infarct size by inhibiting neutrophil-related processes (94). Recently, it was shown in mice that the infarct-sparing effect of adenosine A_{2A} receptor stimulation at reperfusion is due primarily to its action on lymphocytes (95).

In addition to the aforementioned effects of adenosine receptor stimulation, which are mostly immediate and short-lived effects, adenosine receptor stimulation also provides more prolonged protection due to its effect on atherogenesis and angiogenesis. Inflammation plays a pivotal role in atherogenesis (96), and adenosine receptor stimulation has been shown to inhibit the development of atherosclerotic lesions in various animal models (18-20). In addition, adenosine plays a role in angiogenesis, and in modulation of proliferation and death of endothelial cells and vascular smooth muscle cells, which plays a key role in the vascular remodeling that leads to vaso-occlusive diseases (9, 97). There is now good evidence that adenosine A_{2B} receptor stimulation inhibits fibroblast and vascular smooth muscle cell proliferation and even induces apoptosis of these cells

(98, 99), inhibits collagen synthesis by smooth muscle cells (100), and promotes endothelial cell proliferation (101). The net effect would be to facilitate the recovery of blood vessels from injury by inhibiting migration and proliferation of vascular smooth muscle cells into the intima while stimulating re-epithelialization of the endothelium-denuded intima (101). Moreover, by stimulation of proliferation and migration of endothelial cells, both direct and via stimulation of the release of vascular endothelial growth factor, adenosine promotes angiogenesis (97).

PHARMACOLOGICAL MODULATION OF THE CARDIOVASCULAR EFFECTS OF ADENOSINE

Several widely used drugs are known to interfere with the metabolism or receptor binding of adenosine. In the studies presented in this thesis, the adenosine receptor antagonist caffeine and the ENT inhibitor dipyridamole are used as pharmacological tools to study the effects of adenosine in humans *in vivo*.

Dipyridamole is a potent inhibitor of the hENT1, with an 50% inhibitory concentration reported to be 30 nM in isolated cells (102) and 2-3 μM in whole blood (51), and therefore blocks cellular uptake of adenosine. Besides nucleoside transport inhibition, dipyridamole can have alternative mechanisms of action, but for these effects, higher concentrations of dipyridamole are required (103). Inhibition of phosphodiesterase was demonstrable with IC_{50} values of > 10 μM (104), and induction of the release of prostacyclin required even higher concentrations (103). The cardiovascular effects of dipyridamole in healthy humans indeed resemble the hemodynamic profile of adenosine (67, 105): local administration of dipyridamole induces vasodilation (106), and systemic administration of dipyridamole increases heart rate and systolic blood pressure (105, 107, 108), although in some studies systolic blood pressure is not affected (109, 110). Moreover, systemic administration of dipyridamole increases minute ventilation and sympathetic activation (108).

In our studies, we use dipyridamole as a tool to inhibit nucleoside transport, as there are several lines of evidence that the cardiovascular effects of dipyridamole in humans *in vivo*, in the same concentration range as we use in our studies, are indeed solely due to nucleoside uptake inhibition: several studies showed that dipyridamole increases the plasma concentration of endogenous adenosine (109, 111, 112), but these results need to be interpreted with caution because it is complicated to reliably determine adenosine concentrations in whole blood. Secondly, dipyridamole selectively potentiates the vasodilator response to the administration of adenosine, but not the vasodilator response to nitroprusside and acetylcholine (113), suggesting that phosphodiesterase inhibition is not involved in this response. Finally, the hemodynamic and vasodilator effects of dipyridamole can effectively be prevented by the adenosine receptor antagonists theophylline and caffeine (105, 106, 109).

Caffeine is one of the most widely consumed pharmacologically active substances in the world, predominantly derived from dietary sources, such as coffee, tea, cola, and chocolate (114). The actions of caffeine at concentrations achieved in normal human consumption are attributed completely to antagonism of endogenous adenosine at its receptors (115). Caffeine is a non-selective adenosine receptor antagonist, with low K_i values for the A_1, A_{2A} and A_{2B} receptor, but a higher K_i for the A_3 receptor. Alternative actions of caffeine, such as phosphodiesterase inhibition,

or direct release of intracellular calcium, only occur at much higher concentrations (115). In experimental settings it has been shown that the hemodynamic responses to the administration of adenosine are effectively prevented by caffeine (116, 117).

As caffeine is an effective adenosine receptor antagonist already at concentrations reached after regular coffee consumption, it is important, when studying the effects of adenosine in humans *in vivo*, to ensure that all volunteers abstain from caffeine containing beverages for some time before the start of the experiments (118). Indeed, in previous studies, the pressor responses to systemic administration of adenosine could only be demonstrated in subjects who abstained from caffeine consumption for at least 12 or 24 hours (118). In the studies presented in this thesis, all subjects were asked to abstain from caffeine for at least 24 hours before each experiment.

OUTLINE OF THE THESIS

The extracellular concentration of adenosine, and thus the stimulation of adenosine receptors, is determined by formation, transport, and degradation of adenosine (figure 1). Together, the effects of adenosine receptor stimulation can promote protection and repair of cardiovascular tissue (figure 2).

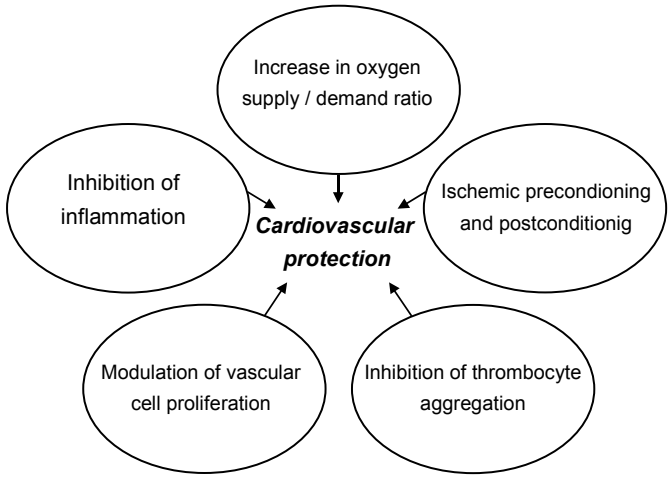

Figure 2: The various effects of adenosine contributing to cardiovascular protection

Interindividual variation in these cardiovascular effects of adenosine could potentially affect individual susceptibility to cardiovascular disease. Previous studies showed that cardiovascular effects of adenosine are indeed subject to large interindividual variation: the vasodilator response to the administration of adenosine into the brachial artery (15 μg/min per dl of forearm tissue) varied from 243% to 1053% in one study (116).

In this thesis, we aimed to study whether genetic variation, metabolic diseases, and pharmacological interventions could modulate the cardiovascular effects of adenosine in humans *in vivo* by interfering with the metabolism or receptor binding of adenosine (figure 3). We focused on

the vasodilator response to the administration of adenosine and the protective effect of adenosine against ischemia-reperfusion injury.

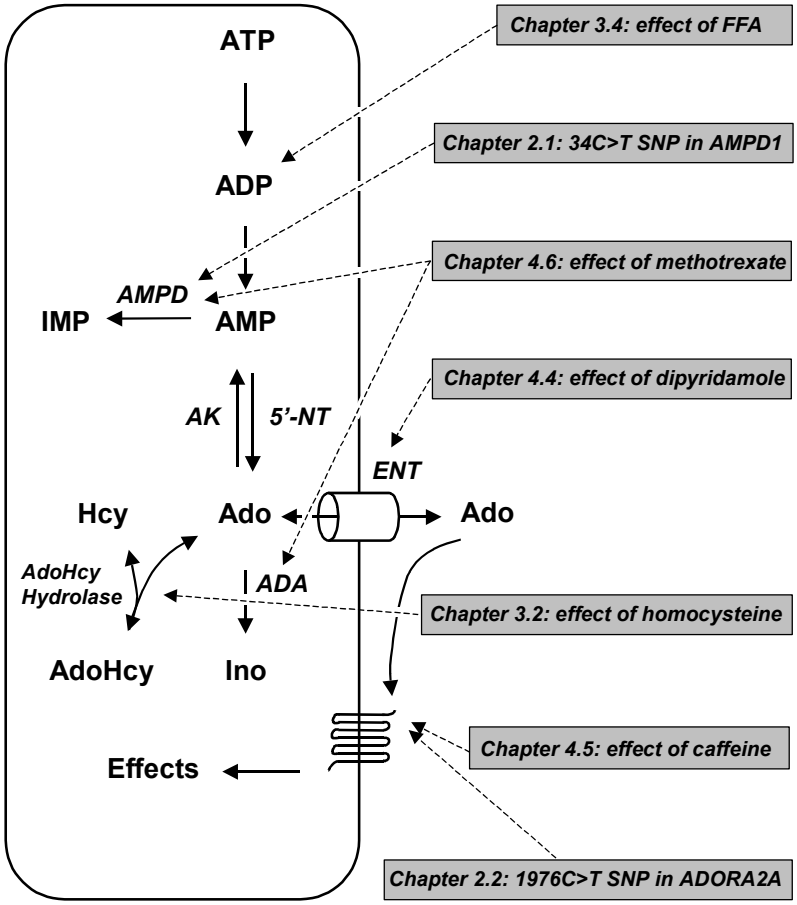

Figure 3: schematic representation of the cellular metabolism of adenosine. The different chapters in this thesis study genetic, metabolic, and pharmacological factors that affect the cardiovascular effects of adenosine by interfering with the metabolism or receptor binding of adenosine, as depicted on the right.

Genetic determinants

A single nucleotide polymorphism (SNP) is defined as a genomic locus where two or more alternative bases occur with appreciable frequency (>1%). SNP's within the coding sequence of a gene could change the amino acid sequence and the biological activity of the protein that is encoded. There are many SNP's in the genes encoding for the proteins involved in the metabolism and receptor activation of adenosine (www.ncbi.nlm.nih.gov/SNP/). Little is known about whether this genetic variation could affect the cardiovascular effects of adenosine. In **chapter 2.1** we focused on the 34C>T variant of the *AMPD1* gene. This variant encodes a severely truncated

protein with loss of function. Previous studies demonstrated that in patients with coronary artery disease, the presence of at least one variant allele is associated with improved survival (21, 119). We hypothesized that in subjects with this variant allele, ischemia-induced adenosine formation is enhanced, which will augment post-occlusive reactive hyperemia and will increase resistance to ischemia-reperfusion. This hypothesis was tested in healthy volunteers. In **chapter 2.2** we explored whether the 1976C>T variant of the adenosine A_{2A} receptor gene (*ADORA2A*) affects the vasodilator response to adenosine. This SNP was selected, as previous neuropsychological studies suggested that this polymorphism could affect the biological function of the A_{2A} receptor (120).

Metabolic determinants

Based on previous *in vitro* studies, we hypothesized that the deleterious cardiovascular sequelae of the metabolic diseases hyperhomocysteinemia and the metabolic syndrome are due to the effects of homocysteine and free fatty acids (FFA), respectively, on the metabolism of adenosine (figure 3). **Chapters 3.1, 3.2, and 3.3** comprise an entire empirical cycle about the potential role of adenosine in the cardiovascular complications of hyperhomocysteinemia: in **chapter 3.1** the hypothesis is discussed that homocysteine reduces the extracellular adenosine concentration by enhanced intracellular formation of S-adenosylhomocysteine, **chapter 3.2** describes the *in vitro* and *in vivo* studies testing the hypothesis, and in **chapter 3.3** this topic is reviewed with incorporation of our own results into the already existing data.

In **chapter 3.4**, we hypothesized that FFA would increase the endogenous adenosine concentration by acting on the adenine nucleotide translocator, which determines the cytosolic ADP/ATP ratio (figure 3). Subsequent adenosine receptor stimulation would induce vasodilation, and reflex activation of the sympathetic nervous system (121). This mechanism could account for the association between the metabolic syndrome (in which plasma concentrations of FFA are typically elevated) and a hyperdynamic circulation. To test this hypothesis, we studied whether the vasodilator effect of FFA could be prevented by the adenosine receptor antagonist caffeine.

Pharmacological modulation of the cardiovascular effects of adenosine

In **chapter 4**, we aimed to determine whether pharmacological modulation of the metabolism and receptor stimulation of adenosine can be used to exploit the beneficial cardiovascular effects of adenosine. In the first series of experiments, we focused on the protective effect of ischemic preconditioning on ischemia-reperfusion injury, as preconditioning can profoundly reduce infarct size in animal studies, and adenosine receptor stimulation has been shown to play a pivotal role in the protective effect of preconditioning (86, 122). We would like to refer to the **chapters 4.1 and 4.2** for a more detailed introduction on ischemic preconditioning. Unfortunately, the protective effect of adenosine is not yet being used in daily clinical practice to reduce ischemia-reperfusion injury in the myocardium for two important reasons. Firstly, until recently, research was focused mainly on the protective effect of adenosine receptor stimulation prior to initiation of the ischemic insult ("preconditioning"). In practice, exploitation of this mechanism is restricted to situations in which cardiac ischemia is carefully planned, such as PTCA or CABG (123). Some recent papers have demonstrated beneficial effects of preconditioning in these settings (124, 125). Recently, however, the concept of "postconditioning" emerged, indicating that also adenosine receptor stimulation at

the onset of reperfusion has the potential to limit ischemia-reperfusion injury (126). A second reason for the discrepancy between the huge amount of preclinical studies on preconditioning and the very low-grade clinical use is the lack of an adequate easy-to-use model to study ischemic preconditioning and the pharmacological modulation of ischemia-reperfusion injury in humans *in vivo*. To this end, we developed and validated ^{99m}Tc-annexin A5 scintigraphy as a novel model to study ischemia-reperfusion injury in the forearm **(chapter 4.3)**. Using this method, we studied whether ischemic preconditioning could reduce injury in the forearm skeletal muscle, induced by ischemic exercise, and whether this protection could be mimicked by the administration of adenosine prior to the ischemic insult **(chapter 4.3)**. In **chapter 4.4** we tested the hypothesis that oral treatment with dipyridamole prior to the ischemic insult would also reduce ischemia-reperfusion injury, as assessed by ^{99m}Tc-annexin A5 scintigraphy. It follows logically that the next chapter tests the hypothesis that caffeine abolishes protection by ischemic preconditioning. This was tested in the forearm in humans *in vivo* as well as *in vitro* in human atrial tissue, obtained during heart surgery **(chapter 4.5)**.

In **chapter 4.6** the potential effect of the anti-rheumatic drug methotrexate on the metabolism of adenosine is addressed. It has been demonstrated previously *in vitro* and in animal studies that the anti-inflammatory effects of methotrexate are mediated by adenosine receptor stimulation, due to inhibition of intracellular degradation of adenosine and increased extracellular formation of adenosine, but it is unknown whether this also holds true for the human *in vivo* situation (127). We hypothesized that methotrexate would potentiate the vasodilator response to adenosine and dipyridamole by these mechanisms.

Finally, in **chapter 4.8** we addressed the potential role of adenosine in the exercise pressor reflex, which is defined as the increase in blood pressure induced by exercise. Until now, it remains controversial whether increased adenosine formation in the exercising muscle and subsequent reflex activation of the sympathetic nervous system is involved in this reflex (128). This is mainly because previous studies suffer from methodological shortcomings, considering the measurement of adenosine in the interstitial compartment during exercise. In this study, we used dipyridamole as a pharmacological tool to study this possible role of adenosine in the pressor reflex to exercise.

TECHNIQUES TO DETERMINE THE CONCENTRATIONS AND EFFECTS OF ADENOSINE IN HUMANS *IN VIVO*

In this thesis, we studied the effect of several interventions on the endogenous adenosine concentration and the cardiovascular effects of adenosine, mainly focused on adenosine-induced vasodilation and adenosine-induced protection against ischemia-reperfusion injury. For practical reasons, we used the forearm vascular bed to study these effects in humans *in vivo*. The techniques used to measure these effects are concisely outlined below.

Microdialysis

It is complicated to reliably determine endogenous adenosine concentrations in plasma because of the extremely short half life of adenosine of approximately 1 second (51). Also, the plasma concentration does not reflect the interstitial concentration of adenosine because the endothelium acts as an active metabolic barrier for adenosine (52, 53). These problems can be circumvented by

using the microdialysis technique (129, 130). A microdialysis probe consists of a double-lumen catheter with a semipermeable membrane on the tip, which is continuously perfused with saline by a microdialysis pump. Because the membrane is permeable for adenosine, but not for the cells and enzymes responsible for its rapid degradation, the adenosine concentration can easily be determined in the dialysate using HPLC. By introducing the microdialysis probe in an antecubital vein or in the forearm skeletal muscle, it is possible to selectively measure the intravascular and interstitial adenosine concentration (57). A disadvantage of this technique is that the absolute adenosine concentration can only be estimated because the equilibrium between the perfusate and the interstitial/intravascular compartment is incomplete. Therefore, the fraction of adenosine recovered across the microdialysis membrane has to be determined by using an internal reference marker or by *in vitro* calibration of the probe in an adenosine solution (57, 58).

Venous occlusion plethysmography

Venous occlusion plethysmography is a safe, and well-validated technique to measure baseline forearm blood flow (FBF), and the local vasodilator responses to the administration of drugs into the brachial artery (131-133). Intrabrachial administration of vaso-active drugs results in a high local concentration in the forearm vascular bed, but prevents significant systemic spillover of the drug and subsequent confounding systemic effects. Repetitive inflation of an upper arm cuff to 40 mmHg induces venous outflow impediment without hindering arterial inflow. The rate of swelling of the forearm, measured with calibrated mercury-in-silastic strain gauges, is used to assess FBF. As blood flow in the hand is predominantly through skin, the circulation of the hand is excluded before each measurement by inflation of a wrist cuff well above systolic arterial pressure. Concomitant measurement of FBF in both arms and continuous measurement of mean arterial pressure in the brachial artery enables detection of the relevant systemic effects on FBF and blood pressure. Calculation of ratio between FBF in the experimental arm and control arm and calculation of forearm vascular resistance (MAP/FBF) can be used to correct for random fluctuations in vascular tone which are independent from the pharmacological intervention, and to correct for small changes in blood pressure, respectively

^{99m}Tc-annexin A5 scintigraphy

There are several human models to study ischemia-reperfusion injury and the modulation of this injury by preconditioning or drugs, such as repeated PTCA and CABG (123). For obvious reasons, these techniques are unsuitable for minimally invasive studies on ischemia-reperfusion injury in healthy subjects. Therefore, we developed ^{99}Tc-annexin A5 scintigraphy as a novel model to study ischemia-reperfusion injury in the forearm in healthy subjects. Annexin A5 is an endogenous protein that binds with high affinity to phosphatidylserine residues in cellular membranes (134). Normally, these residues are located exclusively on the inner leaflet of the cellular membrane, but very early after the onset of ischemia phosphatidylserines are also exposed to the outside of the membrane, either as early sign of apoptosis, or as reversible event, preceding initiation of cellular death pathways (134, 135). By labeling recombinant annexin A5 with ^{99m}Technetium, it is possible to detect these cellular changes in response to ischemia-reperfusion in humans *in vivo* using a gamma camera (136, 137). A more detailed description of the rationale and validation of this technique is given in Chapter 4.3.

CHAPTER 2

GENETIC DETERMINANTS OF THE VASCULAR EFFECTS OF ADENOSINE

CHAPTER 2.1

AUGMENTED HYPEREMIA AND REDUCED TISSUE INJURY IN RESPONSE TO ISCHEMIA IN SUBJECTS WITH THE 34C>T VARIANT OF THE *AMPD1* GENE

Submitted

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ABSTRACT

Aim: In patients with coronary artery disease the 34C>T variant of the Adenosine Mono-Phosphate Deaminase gene *(AMPD1)*, encoding a dysfunctional protein, predicts improved survival. We hypothesized that in subjects with this variant allele, ischemia-induced intracellular adenosine formation is increased, augmenting reactive hyperemia and ischemic tolerance.

Methods and Results: We selected 10 healthy subjects with the CT genotype and 10 CC controls. The forearm vasodilator response to 2 and 5 minutes of ischemia (venous occlusion plethysmography) was higher in the CT group, whereas maximum vasodilation (13 minutes of ischemia) did not differ (25.4±2.5, 32.7±2.2, and 38.6±2.6 ml/min/dl in the CT group versus 21.9±2.2, 28.5±2.4, and 41.0±3.3 ml/min/dl in controls, P=0.03). Additionally, ischemia-reperfusion injury was assessed in thenar muscle using ^{99m}Tc-annexin A5 scintigraphy after forearm ischemic exercise to detect externalized membrane phosphatidylserines. At reperfusion ^{99m}Tc-annexin was administered intravenously. At 1 and 4 hours post-injection, annexin targeting (percentage difference between experimental and contralateral hand) was 5.2±1.8% and 3.5±2.3% in the CT group versus 8.9±3.4% and 9.8±3.9% in controls (n=7; P=0.03).

Conclusion: The 34C>T variant of *AMPD1* augments vasodilation and reduces tissue injury in response to forearm ischemia. These mechanisms could contribute to the survival benefit of cardiovascular patients with this variant allele.

Key Words: adenosine; *AMPD1* genotype; ischemia; blood flow; scintigraphy.

INTRODUCTION

In patients with heart failure and in patients with coronary artery disease, the presence of the 34C>T polymorphism on at least one of the alleles encoding for the enzyme adenosine mono-phosphate deaminase (*AMPD1*) is associated with prolonged survival (21, 119). AMPD is one of the enzymes involved in the intracellular metabolism of the purine nucleoside adenosine (figure 1), catalyzing the conversion of AMP to IMP (25).

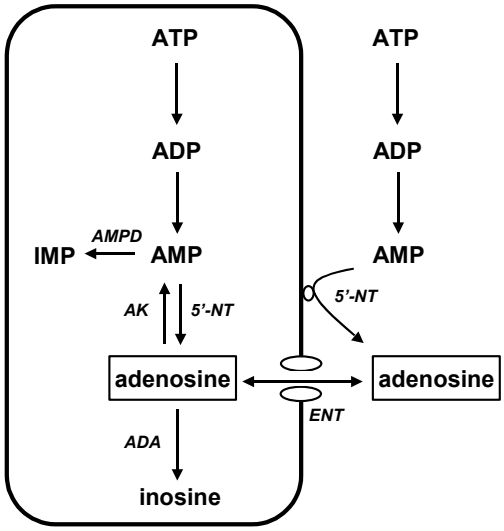

Figure 1: Schematic representation of the formation, transport and degradation of adenosine. ADA: adenosine deaminase; AK: adenosine kinase; IMP: inosine mono-phosphate; 5'-NT: 5'-nucleotidase.

In humans, four AMPD isoforms have been described, named after the source from which they were initially purified: M (muscle), L (liver), E1 and E2 (erythrocyte), encoded by the different genes *AMPD1*, *AMPD2* and *AMPD3* (24). Approximately 15-20% of Caucasian and African American individuals are heterozygous or homozygous for the 34C>T variant of *AMPD1*, encoding a truncated protein with loss of function (138).

Under conditions of impending tissue danger, such as ischemia, the extracellular adenosine concentration rapidly increases (14). Subsequent stimulation of adenosine receptors induces various effects, such as vasodilation, ischemic preconditioning, and inhibition of inflammation (5). Based on the observation that AMPD activity is significantly reduced in patients heterozygous for the 34C>T variant (CT genotype), it was suggested that in these patients during ischemia AMP is preferentially degraded into adenosine, which will increase ischemic tolerance (119).
We hypothesized that subjects with the 34C>T variant show augmented reactive hyperemia and reduced ischemia-reperfusion injury in response to ischemia. To test this hypothesis, we selected healthy subjects with the CT genotype and controls homozygous for the common C allele (CC genotype), but with otherwise similar parameters of adenosine transport and metabolism. The vasodilator response to 2, 5 and 13 minutes of forearm ischemia was determined by venous

occlusion plethysmography. This experiment was repeated in the presence of dipyridamole, as a pharmacological tool to inhibit facilitated diffusion of adenosine through the equilibrative nucleoside transporter (ENT), to explore the role of increased intracellular adenosine formation. We hypothesized that dipyridamole potentiates reactive hyperemia, as previous evidence suggests that during ischemia transport of adenosine over the cellular membrane is mainly from outside the cell inwards (13, 139). This effect of dipyridamole, however, is expected to be less pronounced in the subjects with the CT genotype because in these subjects impaired conversion of AMP to IMP will enhance intracellular adenosine formation, thus decreasing the transmembranous adenosine concentration gradient, which is the driving force for adenosine transport (50).

In an additional series of experiments, ischemia-reperfusion injury was determined in thenar muscle, using ^{99m}Tc-annexin A5 scintigraphy to detect phosphatidylserine exposure on cellular membranes of affected cells as early marker of ischemic injury, as described previously (140).

METHODS

Subjects

The study protocol was approved by the Institutional Review Board of the Radboud University Nijmegen Medical Center. Ninety-six healthy subjects participated after signing informed consent. Blood was drawn for genotyping of the *AMPD1* and of the adenosine A_{2A} receptor gene (*ADORA2A*) and for determination of the activities of the ENT, adenosine kinase and adenosine deaminase. Twenty subjects had the CT genotype and 2 the TT genotype for *AMPD1*. To study reactive hyperemia, we selected 10 subjects with the CT genotype, and 10 control subjects with the CC genotype, but with otherwise similar *ADORA2A* 1976C>T genotype frequencies, and similar activities of the ENT transporter, adenosine kinase and adenosine deaminase. The *ADORA2A* 1976C>T genotype was determined as the TT variant could potentially affect its function (120). To assess tolerance to ischemia-reperfusion injury, we selected 7 male subjects with the CT genotype and 7 controls (table 1).

Post-occlusive reactive hyperemia

Experiments were performed in a temperature-controlled room (23 °C) in the morning after an overnight fast and 24 hours of caffeine abstinence. The brachial artery of the non-dominant arm was cannulated with a 20-gauge catheter for intra-arterial drug administration and blood pressure recording. Forearm blood flow (FBF) was recorded simultaneously at both arms by venous occlusion plethysmography using mercury-in-silastic strain gauges (141). Before each recording a wrist cuff was inflated to 200 mmHg to exclude the hand circulation. Arterial occlusion of the non-dominant forearm was induced by inflation of an additional upper arm cuff to 200 mmHg.

Thirty minutes after insertion FBF was recorded for 5 minutes during saline infusion (baseline FBF). Subsequently, the FBF response to 2, 5 and 13 minutes of forearm ischemia was measured for 3, 5 and 5 minutes, respectively, as described previously (142). During the last minute of the 13 minute period of ischemia the subjects performed rhythmic handgripping, as a stimulus for maximal forearm vasodilation, to exclude any possible structural differences in forearm resistance vessels (143). After 40 minutes of equilibration this experiment was repeated, but now during

concomitant administration of dipyridamole (Persantin®, Boehringer Ingelheim, Espana S.A., Spain) into the brachial artery (7.4 nmol/min per dl of forearm tissue). A previous study demonstrated that this dose does not affect baseline FBF but significantly potentiates adenosine-induced vasodilation (106). Administration of dipyridamole was discontinued 10 seconds after initiation of forearm occlusion and restarted 10 seconds before reperfusion. After 30 minutes of equilibration the FBF response to intrabrachial administration of sodium nitroprusside (SNP; 0.06 and 0.6 μg/min/dl; Pharmacy Radboud University Nijmegen Medical Center) and acetylcholine (ACh; 0.5 and 2.0 μg/min/dl; miochol®-E, Novartis, Bournonville Pharma BV, The Hague, The Netherlands) was recorded for 5 minutes per dose to study endothelium-independent and endothelium-dependent vasodilation, respectively.

Ischemia-reperfusion injury

The circulation to the non-dominant forearm was interrupted for 10 minutes by inflation of an upper arm cuff to 200 mmHg. Concomitantly, subjects performed rhythmic isometric handgripping with a dynamometer at 50% of maximum force, alternating 5 seconds of contraction and 5 seconds of relaxation until exhaustion. Immediately upon reperfusion ^{99m}Tc-annexin A5 was administered intravenously into the dominant arm. At 1 and 4 hours post-injection, both hands were scanned simultaneously until ≥ 150,000 counts were detected (or when a maximum scanning time of 25 minutes was reached) using a gamma camera equipped with a low-energy high resolution collimator (Siemens Orbiter, Hoffman Estates, Illinois, USA) connected to a Hermes Gold image processing system (Nuclear Diagnostics, Stockholm, Sweden) as previously described (140).

Genetic analysis

Blood was drawn in EDTA-containing vacutainers and stored at -70 °C until DNA isolation. Genomic DNA isolation was performed using a standard desalting protocol (144). Genotyping was performed by pyrosequencing according to the protocol of the manufacturer (Pyrosequencing AB, Uppsala, Sweden) (145).

For genotyping of the *AMPD1* 34C>T variant the following reaction was carried out: Primers FW8076 (5'-gcaatctacatgtgtctacc-3', 10 pmol) and RV8077 (5'-*agcgctgctccggttcatagatt*atagccatgtttctgaatta-3', 1 pmol) were combined with a biotinylated universal primer (4205: 5'Biotin-*gctgctccggttcatagatt*-3', 9 pmol), 50 ng of DNA, 0.32 mM dNTPs, 0.5 U Taq DNA polymerase (Invitrogen, Breda, The Netherlands) and a PCR buffer containing 60 mM Tris-HCl pH 8.5, 15 mM ammonium sulfate and 1.5 mM $MgCl_2$ in a total volume of 25 μl. For genotyping of the *ADORA2A* 1976C>T variant the following amplification reaction was carried out: Primers FW8073 (5'-*gacgggacaccgctgatcgtttac*ggaggcccaatggcta-3', 1 pmol) and RV8074 (5'-cccaacgtgactggtcaag-3', 10 pmol) were combined with a biotin-labeled universal primer (4206: 5'Biotin-*gggacaccgctgatcgttta*-3', 9 pmol), 50 ng DNA, 0.32 mM dNTPs, 0.5 U Taq DNA polymerase (Invitrogen, Breda, The Netherlands) and a PCR buffer containing 10 mM Tris-HCL pH 8.0, 50 mM KCl, 0.1% Triton X-100 (v/v), 0.015% gelatin (w/v), 5% DMSO (v/v) and 1.5 mM $MgCl_2$ in a volume of 25 μl. The cycling conditions for both amplification reactions were similar, starting with 5 min at 92°C, followed by 39 cycles (35 in the case of *ADORA2A*) of 1 min 92°C, 1 min at the optimized annealing temperature (55°C for *AMPD1*, 54°C for *ADORA2A*) and 1 min at 72°C, then

followed by an extra 5 min 72°C. The amplifications were performed in a PTC-200 Multicycler (MJ-Research via Biozym, Landgraaf, The Netherlands).
Pyrosequencing for *AMPD1* was performed in a forward assay using primer 8078 (5'-tcatacagctgaagagaaa-3'), for *ADORA2A* in a reverse assay with the primer 8075 (5'-ctcaccagcccca-3').

Laboratory analysis
In isolated erythrocytes, uridine uptake was determined as previously described (146). V_{max} and K_m values for the ENT were estimated according to Michaelis-Menten kinetics. For the determination of adenosine deaminase activity, adenosine was added to erythrocyte lysate in Tris-buffer (0.35% erythrocytes) in a final concentration of 25, 50, 100, 200, and 300 µmol/l at 37 °C. After 15 minutes 50 µl 1.5 M $HClO_4$ was added, and the solution was placed on ice. After centrifugation, 0.5 M trioctylamine in chloroform was added to the supernatant in equal volumes. Finally, after centrifugation, the neutralized supernatant was used for detection of inosine and hypoxanthine with reversed-phase HPLC using tetrabutylammonium hydrogen sulfate (10 mM) as the ion-pair forming agent (pH 6.0) with UV detection set at 254 nm. The sum of inosine and hypoxanthine was used for the calculation of V_{max} and K_m for adenosine deaminase. For the determination of adenosine kinase activity, adenosine was added to 10 µl lysate in 43 µl distilled water, 2 µl 50 mM MgCl, 2 µl 100 mM DTT, 2 µl 50 mM GTP, 0.7 µl 3 mM erythro-9-(2-hydroxynon-3-yl)-adenine (EHNA) and 100 µl Tris-buffer in a final concentration of 0.1, 0.2, 0.5, 1, and 2 µmol/l at 37 °C. After 3.5 minutes 50 µl 1.5 M $HClO_4$ was added, and the solution was placed on ice. After centrifugation, 100 µl 0.5 M trioctylamine in chloroform was added to 150 µl supernatant. Finally, after centrifugation, the AMP concentration in the supernatant was measured with reversed-phase HPLC using tetrabutylammonium hydrogen sulfate (10 mM) as the ion-pair forming agent (pH 7.7) with UV detection set at 260 nm. The increase in AMP was used to calculate V_{max} and K_m values. Plasma caffeine concentration was determined by reversed-phase HPLC with UV detection set at 273 nm (147). All measurements were performed in duplicate.

Preparation of ^{99m}Tc-HYNIC-annexin A5
^{99m}Tc-HYNIC-annexin A5 was prepared as previously described (148). Briefly, recombinant human annexin A5 (kindly provided by Dr. Vanderheijden, Theseus Imaging Corporation, Boston, MA) was conjugated with succinimidyl-hydrazinonicotinic acid (HYNIC). 100 µg samples of the annexin-HYNIC conjugate in 500 µl 113 mM tricine, pH 6.8 were stored at -20 °C. Before each experiment ^{99m}Tc-Pertechnetate (500 MBq) was added to the 100 µg sample of the annexin-HYNIC conjugate in the presence of 50 µg stannous sulphate. 400 MBq ^{99m}Tc-annexin A5 was administered to each subject.

Statistical Analysis
All values are expressed as mean ± SE, unless otherwise specified. Baseline characteristics were compared with a Mann Whitney test. Analysis of the FBF responses was performed off-line blinded for the specific *AMPD1* genotype. For each post-occlusive period maximum FBF was determined. Maximum FBF values for the 3 consecutive ischemic periods were compared by repeated measures ANOVA (SPSS for Windows, release 12.0.1). In the second study, all digital scintigraphic images

were analyzed off-line by the same blinded investigator (WJGO), using Hermes Gold software. Region-of-interest analysis was performed for the thenar muscle region, as described previously (140). Radioactivity was expressed as counts per pixel. To correct for background activity, the final result was expressed as the percentage difference between the experimental and control hand ('targeting'). Because annexin targeting did not show a Gaussian distribution in either group, we used a Mann Whitney test to compare the change in annexin targeting from 1 to 4 hours post-injection between groups.

RESULTS

V_{max} and K_m values of adenosine deaminase, adenosine kinase, and the ENT did not differ between the groups, and *ADORA2A* genotype frequencies were equally distributed (table 1).

Table 1: baseline characteristics of the two groups in both studies (mean ± SD).

Characteristics	Plethysmography study		Scintigraphy study	
	CT genotype	CC genotype	CT genotype	CC genotype
Sex (m/f)	7/3	7/3	7/0	7/0
Age (years)	23 ± 3	23 ± 3	25 ± 4	25 ± 7
Weight (kg)	73 ± 11	73 ± 10	81 ± 13	74 ± 11
SBP (mmHg)	125 ± 8	126 ± 9	125 ± 10	127 ± 4
DBP (mmHg)	71 ± 6	73 ± 7	79 ± 10	71 ± 9
Heart rate (bpm)	71 ± 6	70 ± 14	69 ± 11	67 ± 13
Plasma glucose (mmol/l)	4.8 ± 0.6	4.7 ± 0.7	5.1 ± 0.6	4.8 ± 0.6
Total cholesterol (mmol/l)	4.3 ± 1.0	4.1 ± 0.6	3.9 ± 0.9	4.1 ± 0.5
Plasma caffeine (mg/l)	0.2 ± 0.4	0.2 ± 0.3	0.2 ± 0.2	0.1 ± 0.1
Uridine uptake				
K_m (μM)	173 ± 26	180 ± 27	186 ± 31	185 ± 45
V_{max} (nmol/min/mg)	268 ± 38	287 ± 26	278 ± 49	275 ± 62
AK activity				
K_m (μM)	0.44 ± 0.15	0.53 ± 0.17	0.49 ± 0.08	0.61 ± 0.16
V_{max} (nmol/min/mg)	28 ± 10	27 ± 9	27 ± 8	29 ± 9
ADA act				
K_m (μM)	44 ± 4	44 ± 5	42 ± 5	43 ± 4
V_{max} (nmol/min/mg)	76 ± 38	71 ± 22	78 ± 36	71 ± 27
ADORA2A 1976 genotype	9 CT/1 CC	9 CT/1 CC	3 CT/3CC/1 TT	4 CT/3 CC

Post-occlusive reactive hyperemia

Baseline FBF was not different between both groups (2.2 ± 0.2 ml/min/dl in the CT genotype group and 2.3 ± 0.2 in the CC group; P=0.6). Also, maximum vasodilation in response to 13 minutes of ischemia did not differ between the groups (figure 2). FBF in response to the 2 and 5-minute period of ischemia was higher in the CT genotype group compared with the controls (figure 2,

P=0.02 for interaction between group and duration of ischemia). There were no differences in FBF in the control arm and mean arterial pressure between both groups.

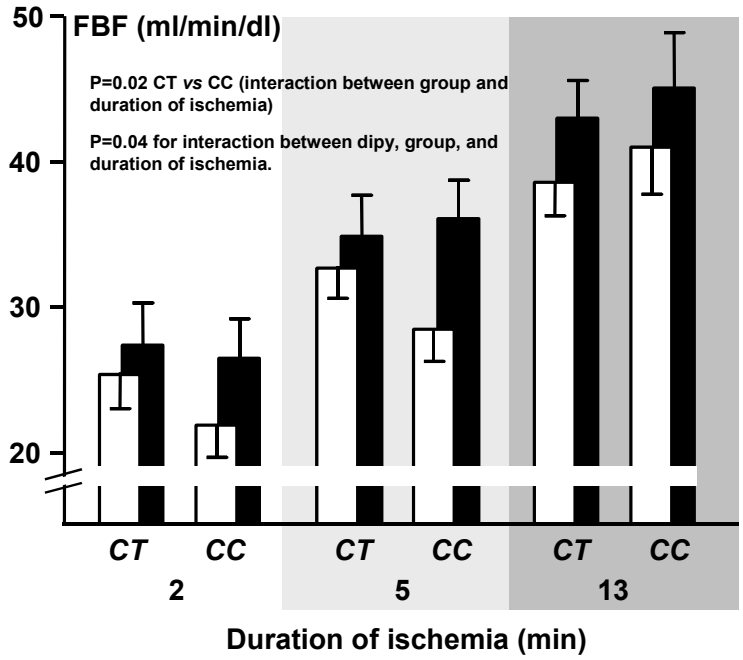

Figure 2: Maximum FBF response to the 3 periods of ischemia in the absence (open bars) and presence (filled bars) of dipyridamole. Reactive hyperemia is higher in the CT genotype group (P=0.02). Dipyridamole potentiates reactive hyperemia in both groups (P=0.04 in the CT genotype group and P=0.001 in the controls). The effect of dipyridamole is less in the CT genotype group (P=0.04).

The effect of administration of dipyridamole on baseline FBF was not different between both groups (from 2.0 ± 0.2 to 2.2 ± 0.3 ml/min/dl in the CT genotype group and from 2.1 ± 0.2 to 2.4 ± 0.2 in the CC group; P=0.3). Dipyridamole potentiated FBF responses to the three periods of ischemia in both groups (figure 2; P=0.04 in the CT genotype group, and P=0.001 in controls). The potentiating effect of dipyridamole was more pronounced in the CC genotype group than in the CT group (figure 2, P=0.04 for interaction between dipyridamole, group and duration of ischemia). As expected, during concomitant administration of dipyridamole, there was no longer any difference in FBF response between both groups (figure 2, P=0.8).

Finally, SNP- and ACh-induced vasodilation did not differ between both groups (figure 3, P=0.7 and P=0.6, respectively).

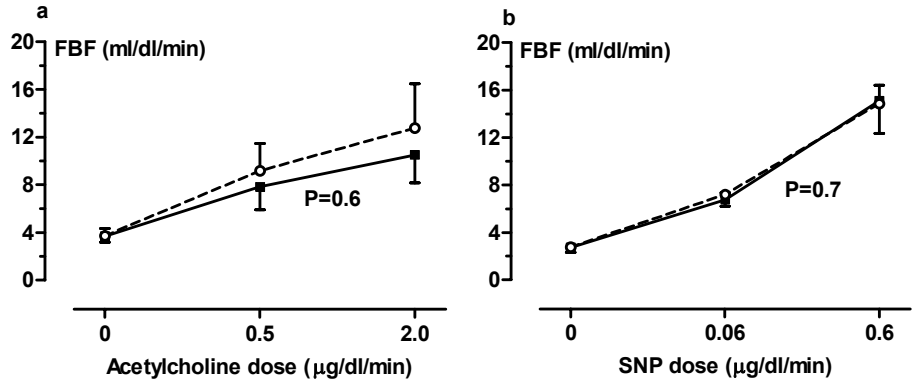

Figure 3: FBF response to intrabrachial administration of acetylcholine (a) and sodium nitroprusside (b) in the CT genotype group (filled squares) and the CC genotype group (open circles).

Ischemia-reperfusion injury

There was no difference in duration of ischemic exercise (154±13 s in the CT genotype group versus 166±15 s in the controls, P=0.5) and maximum contractile force (55±4 kg in the CT genotype group versus 47±3 kg in controls, P=0.2) between both groups.

Annexin targeting was less in the CT genotype group than in CC controls (figure 4; P=0.03).

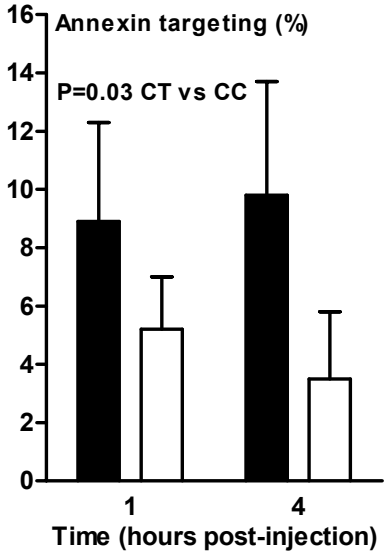

Figure 4: Annexin targeting in the CT genotype group (open bars) and CC control group (filled bars) at 1 and 4 hours post-injection. P=0.03 for difference in the change in annexin targeting from 1 to 4 hours post-injection between the groups.

DISCUSSION

The results of the present study demonstrate that in subjects with the 34C>T variant of *AMPD1*, reactive hyperemia in response to forearm ischemia is enhanced and tissue injury is reduced. These mechanisms could well contribute to the reduced cardiovascular mortality in patients with genotypes containing the variant allele.

Previous studies have shown that the 34C>T variant of *AMPD1* is associated with improved survival in patients with heart failure (119) and in patients with coronary artery disease (21). Also in cardiac donors, the 34C>T variant appears to protect against acute heart failure (149). It is unknown which mechanism is responsible for these beneficial cardiovascular effects. The C→T transition results in a nonsense mutation encoding a truncated AMPD protein (138). AMPD activity in human muscle biopsies is 30-40% of normal in subjects with the CT genotype and less than 2% in subjects homozygous for the variant allele (150). Although predominantly expressed in skeletal muscle, *AMPD1* is also expressed in cardiomyocytes and aorta (25). Indeed, also in cardiac tissue AMPD activity is approximately 50% reduced in patients with the CT genotype (151). Considering the various beneficial cardiovascular effects of adenosine receptor stimulation, it is logical to assume that in subjects with AMPD deficiency, ischemia-induced adenosine formation and subsequent adenosine receptor stimulation is potentiated (figure 1), and that this mechanism is responsible for the improved cardiovascular outcome.

In the present study, we focused on reactive hyperemia as a possible mechanism by which AMPD deficiency could increase resistance to ischemia-reperfusion. This mechanism was already suggested by recent preliminary data in healthy volunteers, demonstrating augmented reactive hyperemia in the forearm, but only in the second minute after reperfusion, and after correction for percent body fat (152). In line with these observations, we showed that reactive hyperemia in response to 2 and 5 minutes of ischemia is augmented in subjects with the CT genotype, compared with a control group, with otherwise similar activities of adenosine deaminase, adenosine kinase, and the ENT, and similar *ADORA2A* genotype frequencies. The groups did not differ in endothelial function. Moreover, the observation that reactive hyperemia in response to a stimulus for maximum forearm vasodilation is similar between both groups, excludes that structural changes of the vessel wall account for the observed differences.

We assume that this augmented reactive hyperemia is mediated by increased adenosine receptor stimulation. Unfortunately, it is extremely difficult to reliably measure adenosine concentrations in humans *in vivo*, because of the short half life of adenosine in blood (<1 second) (51) and because the endothelium acts as a highly active metabolic barrier for adenosine (52). Therefore, the concentration of adenosine in blood does not reflect the concentration of adenosine in the vicinity of adenosine receptors on several cell types, such as endothelial cells, cardiomyocytes, and vascular smooth muscle cells. Several studies have demonstrated that during strenuous exercise the tissue adenosine concentration in skeletal muscle biopsies is increased in subjects with the CT genotype compared with controls, but there are no data on the extracellular adenosine concentration during exercise or ischemia in these subjects (31, 150). Because of these difficulties in measuring adenosine concentration, we previously validated dipyridamole as pharmacological tool to inhibit the ENT.

We demonstrated that systemic administration of dipyridamole (administered orally) significantly inhibited *ex vivo* adenosine uptake in isolated erythrocytes (146). Secondly, during concomitant administration of adenosine into the brachial artery, administration of dipyridamole increased the adenosine concentration in the venous effluent (as measured with microdialysis) (153). Thirdly, in the same dose as in the present study, dipyridamole did not affect baseline vascular tone, but significantly potentiated the vasodilator response to the intrabrachial administration of adenosine (154). Finally, the vasodilator response to intrabrachial administration of dipyridamole and the hemodynamic responses to the systemic administration of dipyridamole could significantly be reduced by adenosine receptor antagonists (105, 106). In the present study, we used this property of dipyridamole to explore the contribution of facilitated adenosine transport to the observed difference in reactive hyperemia between subjects with the CT genotype and controls.

It is well known that during ischemia, the extracellular adenosine concentration rapidly increases (14, 155). In the present study, dipyridamole potentiated reactive hyperemia in both groups. This finding indicates that during ischemia and early reperfusion flux of adenosine over the cellular membrane is mainly directed inwards. In subjects with the CT genotype the potentiating effect of dipyridamole on maximum reactive FBF was less than in controls. This observation suggests that in these subjects, during ischemia and early reperfusion the transmembranous adenosine gradient is less than in control subjects. This is compatible with the view that during ischemia in subjects with decreased activity of the cytosolic AMPD enzyme, intracellular - and not extracellular - formation of adenosine from AMP is increased.

Adenosine receptor stimulation increases resistance to ischemia-reperfusion by several mechanisms (8). We hypothesized that in patients with the 34C>T *AMPD1* variant, augmented adenosine formation during ischemia not only augments reactive hyperemia, but also reduces ischemia-reperfusion injury. To test this hypothesis, we used ^{99m}Tc-annexin A5 scintigraphy to determine ischemia-reperfusion injury in thenar muscle. We demonstrated that ischemia-reperfusion injury is attenuated in subjects with the 34C>T genotype. ^{99m}Tc-annexin A5 scintigraphy is based on the high affinity binding of annexin A5 to phosphatidylserine residues on cellular membranes. It is well documented that loss of membrane asymmetry is an early general feature of apoptosis, resulting in externalization of phosphatidylserine residues in affected cells, thus providing binding sites for annexin A5 (134). By labeling recombinant annexin A5 with ^{99m}Technetium it is possible to detect these cellular changes *in vivo* with a gamma camera (156). In previous studies, we validated ^{99m}Tc-annexin A5 scintigraphy as a sensitive model to detect ischemia-reperfusion injury in skeletal muscle, and demonstrated that this injury could be reduced by administration of adenosine and dipyridamole prior to the ischemic insult (140, 146). Also, we demonstrated that the adenosine receptor antagonist caffeine abolishes protection by ischemic preconditioning (148).

Several potential limitations of our study should be addressed. First, we only studied subjects who were heterozygous for the 34C>T variant. In subjects with the TT genotype reactive hyperemia and ischemic tolerance could be augmented even more. Second, for technical reasons, we did not measure plasma adenosine concentrations. However, the observation that the effect of dipyridamole on reactive hyperemia was less in subjects with the CT genotype provides evidence

that indeed intracellular adenosine formation during ischemia is enhanced in these subjects. Finally, we studied the response to ischemia in the forearm, and not in the heart. However, the 34C>T variant of *AMPD1* also reduces AMPD activity in cardiac tissue (151).

In conclusion, we demonstrated that the 34C>T variant of the *AMPD1* gene augments post-occlusive reactive hyperemia and tolerance to ischemia-reperfusion. The reduced potentiating effect of dipyridamole on reactive hyperemia in subjects with the CT genotype indicates that the *AMPD1* gene determines the effect of dipyridamole, and supports the concept that the transmembranous adenosine concentration gradient during ischemia is diminished in these subjects, which is compatible with increased intracellular formation of adenosine during ischemia. Our observations predict that in trials with ENT-inhibitors designed to explore the protective effect of endogenous adenosine against ischemia-reperfusion injury, most benefit is expected in patients without the 34C>T variant allele. The augmented reactive hyperemia and increased ischemic tolerance could contribute to the previously observed improved survival in cardiovascular patients with this variant allele and bridges the gap between the rational theoretical concept of adenosinergic cardiovascular protection and previous epidemiological observations.

ACKNOWLEDGEMENTS

NP Riksen is recipient of an AGIKO-fellowship of the Netherlands Organization for Scientific Research (ZonMw). The contribution of GA Rongen has been made possible by a fellowship from the Royal Netherlands Academy of Arts and Sciences (KNAW). The authors would like to thank Joke Verpalen and Marlies Naber for the laboratory experiments.

CHAPTER 2.2

THE 1976C>T POLYMORPHISM IN THE ADENOSINE A_{2A} RECEPTOR GENE DOES NOT AFFECT THE VASODILATOR RESPONSE TO ADENOSINE IN HUMANS *IN VIVO*

Pharmacogenet Genomics (in press)

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ABSTRACT

The 1976C>T polymorphism in the adenosine A_{2A} receptor gene *(ADORA2A)* modulates the psychological response to administration of the adenosine receptor antagonist caffeine. We quantified the vascular response to adenosine and caffeine to determine the relevance of this variant allele in the physiological response to these agents. We selected 10 subjects with the TT genotype and 10 CC controls, with otherwise similar activities of other proteins involved in the metabolism of adenosine. The vasodilator response to the intrabrachial administration of adenosine (0.5, 1.5, 5.0, 15.0, and 50.0 μg/min/dl; venous occlusion plethysmography) was not different between the groups (P=0.4). Also, the effect of subsequent administration of caffeine (90 μg/min/dl) was not different (P=0.7). We conclude that the 1976C>T polymorphism does not affect the vascular response to adenosine and caffeine in humans *in vivo*. Therefore, this polymorphism does not contribute to the variation in the effects of adenosine receptor stimulation.

Key Words: Adenosine A_{2A} Receptor; Polymorphism; Vasodilation; Caffeine

INTRODUCTION

In situations of impending tissue danger, such as during ischemia or inflammation, the local extracellular adenosine concentration rapidly increases, and subsequent stimulation of adenosine A_{2A} receptors induces various cardiovascular effects, which have the potential to limit myocardial infarct size and attenuate the development of atherosclerosis (18, 95).

In the brain, the adenosine A_{2A} receptor has been implicated in the regulation of mental arousal (69). The common 1976C>T polymorphism in the adenosine A_{2A} receptor gene (*ADORA2A*) has been shown to modulate the psychological response to the systemic administration of the adenosine receptor antagonist caffeine (120), and has been associated with panic disorder (157). These observations suggest that this polymorphism alters A_{2A} receptor function which may have implications for the action of adenosine and caffeine in other organs, including the cardiovascular system.

In this study, we explored whether this variant allele also affects the vascular effects of adenosine and caffeine in humans *in vivo.* To this end, we measured adenosine-induced vasodilation, as this is mediated by adenosine A_{2A} receptor stimulation (158). We assessed the vasodilator response to the administration of adenosine into the brachial artery in healthy volunteers homozygous for the 1976C>T variant (TT genotype) and controls homozygous for the common C allele (CC genotype). Subsequently, we investigated the effect of caffeine on adenosine-induced vasodilation. As the metabolism of adenosine could also affect the vasodilator response to adenosine (159), we also determined the activities of adenosine deaminase, adenosine kinase, the equilibrative nucleoside transporter (ENT), and the adenosine monophosphate (*AMPD1*) genotype.

METHODS

The protocol was approved by the Institutional Review Board of the Radboud University Nijmegen Medical Center. After signing for informed consent, blood was drawn from 96 healthy subjects for genotyping and assessment of the aforementioned protein activities. 10 subjects with the *ADORA2A* 1976TT genotype and 10 CC controls were selected to participate.

Genetic analysis

After genomic DNA isolation from blood (144), genotyping of the 1976C>T polymorphism in *ADORA2A* and the 34C>T polymorphism in *AMPD1* was performed by pyrosequencing (145) according to the manufacturer's protocol (Pyrosequencing AB, Uppsala, Sweden).
For genotyping of the *ADORA2A* 1976C>T variant primers FW8073 (5'-*gacgggacaccgctgatcgttta*cggaggcccaatggcta-3', 1 pmol) and RV8074 (5'-cccaacgtgactggtcaag-3', 10 pmol) were combined with a biotin-labeled universal primer (4206: 5'Biotin-*gggacaccgctgatcgttta*-3', 9 pmol), 50 ng DNA, 0.32 mM dNTPs, 0.5 U Taq DNA polymerase (Invitrogen, Breda, The Netherlands) and a PCR buffer containing 10 mM Tris-HCl pH 8.0, 50 mM KCl, 0.1% Triton X-100 (v/v), 0.015% gelatin (w/v), 5% DMSO (v/v) and 1.5 mM $MgCl_2$ in a volume of 25 µl. For genotyping of the *AMPD1* 34C>T variant only the primers (FW8076 (5'-gcaatctacatgtgtctacc-3', 10 pmol), RV8077 (5'-*agcgctgctccggttcatagatt*atagccatgtttctgaatta-3', 1 pmol), universal primer (4205: 5'Biotin-*gctgctccggttcatagatt*-3', 9 pmol)) and the PCR buffer (60 mM Tris-HCl pH 8.5, 15 mM

ammonium sulfate and 1.5 mM $MgCl_2$) differed. Cycling conditions for both amplification reactions (in a PTC-200 Multicycler; MJ-Research via Biozym, Landgraaf, The Netherlands) were also similar, starting with 5 min at 92°C, followed by 35 cycles (39 for *AMPD1*) of 1 min 92°C, 1 min at the optimized annealing temperature (54°C for *ADORA2A*, 55°C for *AMPD1*) and 1 min at 72°C, then extra final 5 min at 72°C. Pyrosequencing of *ADORA2A* was performed in a reverse assay with the primer 8075 (5'-ctcaccagcccca-3'), of *AMPD1* in a forward assay using primer 8078 (5'-tcatacagctgaagagaaa-3').

Laboratory analysis

The ENT transporter was characterized in isolated erythrocytes by quantifying uridine uptake, as previously described (146). To assess adenosine deaminase activity, adenosine was added to erythrocyte lysate in Tris-buffer (0.35% erythrocytes) in a final concentration of 25, 50, 100, 200, and 300 μmol/l at 37 °C. After 15 minutes 50 μl 1.5 M $HClO_4$ was added and, after centrifugation, 0.5 M trioctylamine in chloroform was added to the supernatant in equal volumes. Finally, the neutralized supernatant was used for detection of inosine and hypoxanthine with reversed phase HPLC. The sum of inosine and hypoxanthine was used for the calculation of V_{max} and K_m.

To assess adenosine kinase activity, adenosine was added to 10 μl erythrocyte lysate in 43 μl distilled water, 2 μl 50 mM MgCl, 2 μl 100 mM DTT, 2 μl 50 mM GTP, 0.7 μl 3 mM erythro-9-(2-hydroxynon-3-yl)-adenine (EHNA) and 100 μl Tris-buffer in a final concentration of 0.1, 0.2, 0.5, 1, and 2 μmol/l at 37 °C. After 3.5 minutes 50 μl 1.5 M $HClO_4$ was added and, after centrifugation, 100 μl 0.5 M trioctylamine in chloroform was added to 150 μl supernatant. Finally, the AMP concentration in the supernatant was measured with reversed phase HPLC and used to calculate V_{max} and K_m values.

Study protocol

All experiments were performed in a temperature-controlled room (23 °C) in the morning after an overnight fast and at least 24 hours of caffeine abstinence. The brachial artery of the non-dominant arm was cannulated with a 20-gauge catheter for drug administration and blood pressure recording, as previously described (116). Forearm blood flow (FBF) was recorded simultaneously on both arms by venous occlusion plethysmography using mercury-in-silastic strain gauges. Before each recording, a wrist cuff was inflated to 200 mmHg to exclude the hand circulation.

Thirty minutes after cannulation, baseline FBF was recorded, followed by the intrabrachial administration of adenosine (0.5, 1.5, 5.0, 15.0, and 50.0 μg/min/dl; 5 min per dosage; FBF values of last 2 minutes averaged to one value). After 30 minutes washout, adenosine was administered in a dose of 5 μg/min/dl and after 5 minutes caffeine was co-infused into the brachial artery for 10 minutes (90 μg/min/dl). We previously demonstrated that, in this dose, caffeine effectively attenuates the vasodilator response to adenosine (116). We also recorded the FBF responses to the administration of sodium nitroprusside (SNP; 0.06 and 0.6 μg/min/dl) and acetylcholine (ACh; 0.5 and 2.0 μg/min/dl) to document possible confounding by differences in endothelial function. Finally, we determined maximum forearm vasodilation (induced by 13 minutes of forearm ischemia with concomitant rhythmic handgripping exercise during the last minute of ischemia), which is dependent only on the structural characteristics of resistance vessels (143).

Statistical Analysis

All values are expressed as mean ± SE, unless specified otherwise. Baseline characteristics were compared with a Mann-Whitney test and FBF values with an ANOVA for repeated measures (SPSS for Windows, release 12.0.1).

RESULTS

Both groups had similar baseline characteristics, protein activities, and *AMPD1* genotype frequencies (table 1).

Table 1: Baseline characteristics of the groups (mean ± SD)

Characteristics	TT genotype	CC genotype
Sex (m/f)	2 / 8	3 / 7
Age (years)	22 ± 2	23 ± 1
Weight (kg)	65 ± 8	64 ± 10
Height (cm)	174 ± 6	172 ± 8
SBP (mmHg)	129 ± 9	123 ± 7
DBP (mmHg)	78 ± 9	77 ± 8
Heart rate (bpm)	72 ± 14	72 ± 7
Plasma caffeine (mg/l)	0.1 ± 0.1	0.5 ± 0.2
AMPD1 genotype	9 CC / 1 CT	9 CC / 1 CT
Uridine uptake		
K_m (μM)	190 ± 37	186 ± 39
V_{max} (nmol/min/mg)	284 ± 41	298 ± 37
Adenosine kinase activity		
K_m (μM)	0.53 ± 0.16	0.51 ± 0.12
V_{max} (nmol/min/mg)	28 ± 8	28 ± 7
Adenosine deaminase activity		
K_m (μM)	44 ± 4	44 ± 5
V_{max} (nmol/min/mg)	86 ± 28	78 ± 22

The vasodilator response to adenosine was not different between the two groups (P=0.4, n=10; figure 1). Likewise, the effect of co-administration of caffeine on the vasodilator response to adenosine did not differ: caffeine infusion changed FBF from 7.8 ± 1.1 ml/min/dl to 7.3 ± 1.1 in the TT genotype group and from 6.4 ± 1.2 to 6.3 ± 1.3 in the controls (P=0.7).

Administration of SNP increased FBF from 2.6 ± 0.3 to 5.0 ± 0.5 and 10.2 ± 1.5 ml/min/dl in subjects with the TT genotype and from 2.2 ± 0.5 to 4.7 ± 0.8 and 12.2 ± 1.7 in control subjects (P=0.2). ACh increased FBF from 3.0 ± 0.4 to 9.0 ± 1.8 and 10.3 ± 2.2 in subjects with the TT genotype and from 2.4 ± 0.7 to 6.0 ± 1.6 and 8.7 ± 1.5 in controls (P=0.6). Finally, maximum FBF was not different between both groups (42 ± 3 ml/min/dl in the TT genotype group versus 42 ± 1 ml/min/dl in CC controls; n=10; P=1.0).

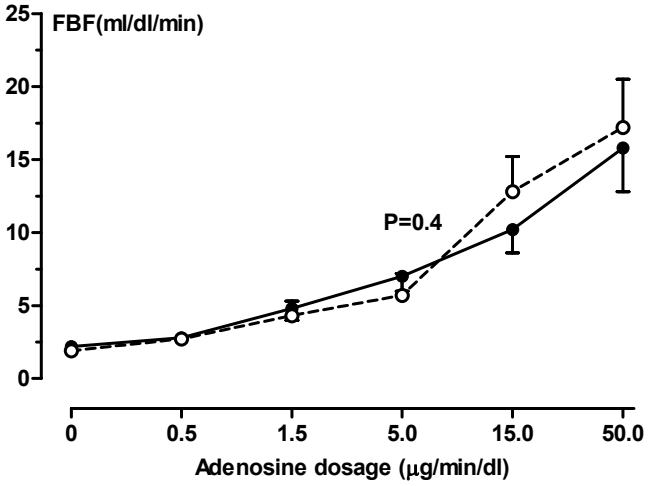

Figure 1: The FBF in response to the administration of incremental dosages of adenosine into the brachial artery in subjects with the 1976TT genotype of the *ADORA2A* (filled circles) and in subjects with the CC control genotype (open circles). P-value represents the difference between both groups.

DISCUSSION

In the present study, we demonstrated in a pharmacological vasodilator dose-response study in humans *in vivo* that the 1976C>T polymorphism in *ADORA2A* does not affect the vascular response to adenosine and caffeine. The TT genotype and CC genotype groups had similar activities of adenosine deaminase, adenosine kinase, the ENT, and similar *AMPD1* 34C>T genotype frequencies, and we also controlled for endothelial function and for maximum vasodilation, to exclude any structural differences in resistance arteries.

It has been shown previously that subjects with the TT genotype had an increased anxiety response to the systemic administration of caffeine (120). Moreover, this variant allele has been associated with panic disorder (157). In addition to the central nervous system, the adenosine A_{2A} receptor is of major importance in the cardiovascular system: the A_{2A} receptor is involved in the vasodilator response to adenosine (158), in the anti-inflammatory effects of adenosine (93), and in the attenuation of atherosclerotic plaque formation (18). Moreover, when activated during reperfusion, adenosine A_{2A} receptors reduce myocardial infarct size in animal models (95). As such, interindividual variation in the function of this receptor could potentially affect cardiovascular risk. It has been shown previously that variation in the metabolism of adenosine could modulate cardiovascular risk: a common nonsense mutation in *AMPD1*, predicting augmented adenosine formation during ischemia, is associated with prolonged survival in patients with coronary artery disease (21).

In the present study, we investigated the effect of the 1976C>T variant in *ADORA2A* on the vascular effects of adenosine and caffeine. We studied vasodilation, as one of the effects of

adenosine A_{2A} receptor stimulation, since there is significant interindividual variation in the vasodilator response to adenosine (116). In a well-controlled human *in vivo* study, we demonstrated that this variant allele does neither affect the vasodilator response to adenosine, nor the effect of subsequent administration of caffeine. Therefore, this genetic variant in *ADORA2A* does not account for the previously observed substantial interindividual variation in adenosine-induced vasodilation and probably, more in general, in cardiovascular risk.

ACKNOWLEDGEMENTS

NP Riksen is recipient of an AGIKO-fellowship of the Netherlands Organization for Scientific Research (ZonMw). The contribution of GA Rongen has been made possible by a fellowship from the Royal Netherlands Academy for Arts and Sciences (KNAW). The authors would like to thank Joke Verpalen and Marlies Naber for the laboratory experiments.

CHAPTER 3

ROLE OF ADENOSINE IN THE CARDIOVASCULAR COMPLICATIONS OF METABOLIC DISEASES

CHAPTER 3.1

POTENTIAL ROLE FOR ADENOSINE IN THE PATHOGENESIS OF THE VASCULAR COMPLICATIONS OF HYPERHOMOCYSTEINEMIA

Cardiovasc Res 2003;59:271-6

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ABSTRACT

Hyperhomocysteinemia is an independent risk factor for cardiovascular disease. Most previous investigations focused on the role of homocysteine as direct pathogenetic factor for these adverse vascular events. However, the exact pathophysiological mechanism is still unknown. In this review we discuss the hypothesis that a decreased extracellular concentration of adenosine could contribute to the adverse cardiovascular effects of hyperhomocysteinemia. Fundamental to this hypothesis is that, *in vivo*, any increase in the plasma concentration of homocysteine reflects an increased intracellular homocysteine concentration, which inevitably will result in a decrease in the adenosine concentration. In this situation, the hydrolase reaction catalysed by S-adenosylhomocysteine hydrolase will reverse and S-adenosylhomocysteine will accumulate at the expense of adenosine.

Stimulation of adenosine receptors by adenosine results in various cardio- and vasoprotective actions, like modulation of vascular resistance, presynaptic inhibition of norepinephrine release, ischemic preconditioning, inhibition of platelet aggregation, modulation of inflammation and regulation of vascular cell proliferation and death. In this respect, a decrease in the adenosine concentration could contribute significantly to the cardiovascular effects of hyperhomocysteinemia.

Keywords: Hyperhomocysteinemia; Adenosine; S-adenosylhomocysteine hydrolase; Pathophysiology; Atherosclerosis.

INTRODUCTION

Patients with homocystinuria have severe hyperhomocysteinemia and are particularly prone to occlusive vascular disease at young age (160). Over the last two decades it became evident that even a mildly elevated plasma concentration of homocysteine is an independent risk factor for cardiovascular disease (161).

Up to now, the underlying mechanism causing the cardiovascular complications in hyperhomocysteinemia is poorly understood. Most investigators in the field focused on the role of homocysteine itself and, more recently, of S-adenosylhomocysteine (162). In this review we discuss the hypothesis that a decreased extracellular concentration of the endogenous nucleoside adenosine could contribute to the pathogenesis of the adverse vascular effects of hyperhomocysteinemia. Fundamental to this hypothesis is that, *in vivo*, any increase in the plasma concentration of homocysteine will result in a decrease in the adenosine concentration. Since adenosine is a potent cardio- and vasoprotective substance, a decrease in the plasma concentration may contribute to the adverse cardiovascular sequelae of hyperhomocysteinemia.

ROLE OF HOMOCYSTEINE AND S-ADENOSYLHOMOCYSTEINE

Since McCully, in 1969, linked elevated plasma homocysteine concentrations with vascular disease (163), many investigations have been conducted to define the causes of atherothrombotic complications in hyperhomocysteinemia. However, up to now, no generally accepted mechanism has been described. Most investigations focused on the role of homocysteine itself.

According to most investigators, endothelial cell injury caused by hyperhomocysteinemia plays a key role in the pathogenesis of cardiovascular complications (reviews: (162, 164)). *In vivo*, hyperhomocysteinemia rapidly impairs endothelial function (165, 166). Many *in vitro* studies have presented evidence of effects of homocysteine on platelets, endothelial cells, vascular smooth muscle cells and on coagulation (review: (162)). However, these studies were not able to provide conclusive mechanisms for the vascular damage associated with hyperhomocysteinemia. Some reports showed that even millimolar concentrations of homocysteine do not induce platelet aggregation *in vitro* (167). Further, many publications which did point towards direct vasotoxic or thrombogenic effects of homocysteine *in vitro* are hardly relevant to the clinical situation. Often, these studies used very high concentrations reduced homocysteine (1 to 10 mM). This is highly unphysiological because total homocysteine concentrations are most often between 10 and 30 µmol/l in plasma of patients with mild hyperhomocysteinemia. Moreover, in blood almost all homocysteine is present in its oxidized disulfide form and only a very small amount of 1 %, or even less, of the total pool of blood homocysteine, is present in its reduced form (168). This means that 10 mM of homocysteine *in vitro* is about 50,000 times the plasma level normally occurring in patients with mild hyperhomocysteinemia (169).

In summary, no coherent, generally accepted view has emerged to explain the pathophysiology of atherosclerotic and thrombotic complications in hyperhomocysteinemia. One potential explanation for these inconsistent results is that not hyperhomocysteinemia itself causes vascular damage, but that high levels of homocysteine are merely a marker for another risk factor. Recently, several studies have provided evidence that not homocysteine, but S-adenosylhomocysteine (AdoHcy), may play an important role in the vascular complications of hyperhomocysteinemia (170-173).

When concentrations of homocysteine are high, the hydrolase reaction catalysed by the AdoHcy hydrolase reverses and AdoHcy is formed from homocysteine and adenosine. AdoHcy in turn is a potent inhibitor of transmethylation reactions in the cell (23, 174). Interestingly, a recent case-control study showed that plasma AdoHcy was a more sensitive marker of cardiovascular disease than plasma homocysteine (175). Wang *et al.,* showed that incubation of cultured endothelial cells with exogenous homocysteine, only in the presence of adenosine, increases intracellular levels of AdoHcy and inhibits carboxymethylation of $p21^{ras}$, resulting in a decreased cellular proliferation (170).

So we can conclude that hyperhomocysteinemia is an independent risk factor for cardiovascular disease and that endothelial dysfunction is the most important mediating mechanism. A direct role for homocysteine itself in the pathophysiology of cardiovascular complications, however, has not been convincingly shown. In conformity with the hypothesis that there might be other risk factors, associated with high levels of homocysteine, for the development of cardiovascular complications, we now focus on the possible role of adenosine in this pathogenesis.

FORMATION AND METABOLISM OF HOMOCYSTEINE IN MAN

Figure 1 shows the biochemical reactions of homocysteine formation, which are relevant to the current discussion. During the conversion of methionine to homocysteine, first S-adenosylmethionine is formed with the use of adenosine-5-triphosphate (ATP). In the human body, S-adenosylmethionine is the ultimate methyl donor of many vital reactions, like DNA, RNA, protein and phospholipid methylation. After demethylation, AdoHcy is generated and further hydrolyzed by the enzyme AdoHcy hydrolase to homocysteine and adenosine. So, the net balance shows that methionine and ATP are converted to homocysteine, a methyl group and adenosine. This pathway may significantly contribute to the production of adenosine in man (left panel of figure 1). Metabolic balance studies in normal adults have shown that the generation of adenosine from AdoHcy is about 20 mmol per day (176). Several *in vitro* studies have quantified the rate of adenosine production from this transmethylation pathway in rat and guinea pig heart (38, 177-180). It was shown that during normoxia, AdoHcy hydrolysis significantly contributes to the intracellular adenosine production (up to one third of total). During hypoxia, however, adenosine is predominantly derived from enhanced hydrolysis of adenosine-mono-phosphate (AMP). It should be realized that the contribution of the transmethylation pathway to adenosine production can differ significantly between various organ systems (23).

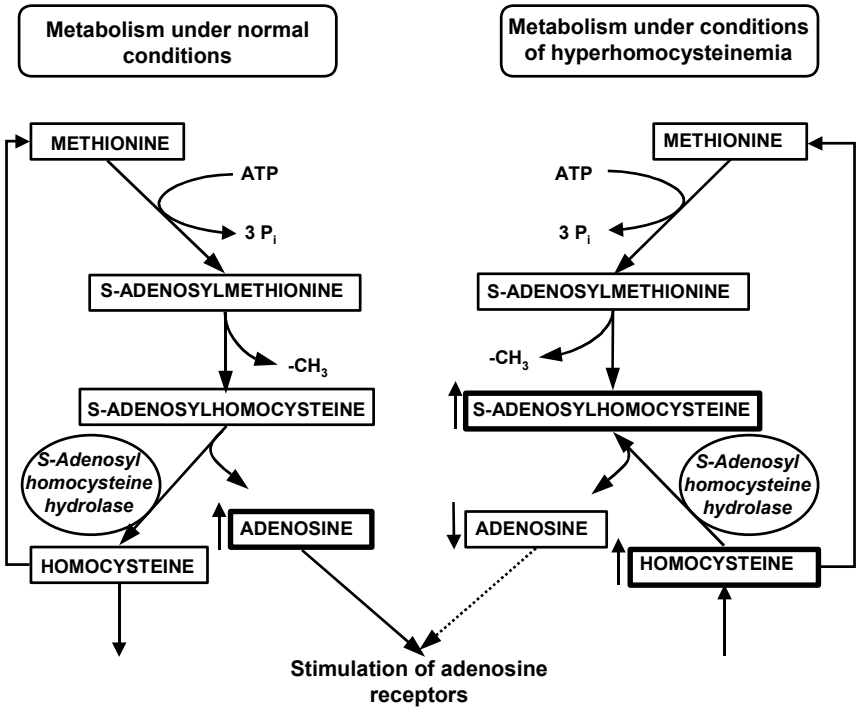

Figure 1: Schematic representation of the biochemical reactions, which are relevant to the current discussion. Under normal conditions, adenosine is formed from the hydrolysis of AdoHcy in man (left part of the diagram). The extracellular concentration contributes to the homeostasis of the vascular system by stimulation of specific adenosine receptors leading to a variety of effects.
Under conditions of increased concentrations of homocysteine, the reaction catalysed by AdoHcy hydrolase will function in the opposite direction (right part of the diagram).

ADENOSINE IN HYPERHOMOCYSTEINEMIA

Of all enzymes in the aforementioned metabolic pathway, only AdoHcy hydrolase is able to function in both directions (23, 162). In fact, the equilibrium constant of the reaction catalyzed by AdoHcy hydrolase favors AdoHcy formation. Nonetheless, the reaction is driven in the opposite direction because the products homocysteine and adenosine are both rapidly removed in the normal *in vivo* situation, causing the enzyme to function in its cleavage direction. However, in case of increased levels of homocysteine, AdoHcy will accumulate at the expense of adenosine (right panel of figure 1). Under these circumstances, intracellular adenosine formation is hampered, increasing the transmembrane adenosine concentration gradient that exists under normoxic conditions (50), and, consequently, the extracellular adenosine concentration is decreased.

It is essential to realize that under baseline normoxic conditions, adenosine is produced continuously in several cell types, originating from two different pathways. First the hydrolysis of AdoHcy by AdoHcy hydrolase. A second important pathway in adenosine formation is the intra-

and extracellular hydrolysis of AMP by 5'-nucleotidase. Under hypoxic conditions, the hydrolysis of AMP greatly increases and predominates the other pathway. Under normoxic conditions, adenosine is quickly taken up by neighbouring cells via nucleoside transporters and is degraded to inosine by adenosine deaminase or rephosphorylated by adenosine kinase (181). As such, the plasma concentration of adenosine is determined by the sum of these processes, and a fall in the formation of adenosine originating from the hydrolysis of AdoHcy should inevitably result in a lower plasma adenosine level, provided that other routes of adenosine metabolism remain constant.

Indeed, several studies showed that the administration of homocysteine or homocysteine thiolactone decreases adenosine release (182, 183). In the study by Sciotti and van Wylen, microdialysis probes were used to measure the adenosine concentration in cerebral interstitial fluid (ISF) in a rat model under different conditions (182). This study showed that infusion of homocysteine thiolactone in the probe, in concentrations of 10^{-5}-10^{-3} M, decreases basal ISF adenosine concentration and cerebral blood flow. Moreover, it showed that homocysteine thiolactone attenuates the increase in dialysate adenosine during ischemia. A comparable experiment was conducted in perfused isolated guinea-pig hearts, in which under hypoxic conditions, homocysteine thiolactone was shown to decrease the adenosine concentration in the perfusate by more than 50%, while the tissue content of AdoHcy increased significantly (183). However, not all studies on this subject showed similar unequivocal results. Deussen *et al* found attenuation of adenosine release with homocysteine during hypoxia, but were not able to show an effect of homocysteine on coronary flow during hypoxia (184). Also, in the dog heart, homocysteine thiolactone was shown not to influence reactive hyperemia, which is believed to be mediated by adenosine (185). This discrepancy could be explained by the multitude of mechanisms, other than adenosine, which contribute to reactive hyperemia (186). Although these studies convincingly illustrated the mechanism we described of homocysteine-induced decrease in adenosine concentration, it has to be emphasized that in these experiments supraphysiological homocysteine concentrations were used and that they do not mimic the situation of hyperhomocysteinemia in man. Moreover, these studies showed that, although the contribution of AdoHcy hydrolysis to basal adenosine formation is limited, under hypoxic conditions (when adenosine production is mostly from AMP hydrolysis) and with high homocysteine levels, this pathway can contribute significantly to intracellular adenosine trapping. Very recently, Chen *et al* found that acute as well as chronic hyperhomocysteinemia in rat decreases plasma and renal interstitial adenosine concentration through the inhibition of AdoHcy hydrolase (187). This study more closely resembled the human situation of hyperhomocysteinemia, in that a plasma homocysteine concentration of approximately 15 μM was induced.

EFFECTS OF ENDOGENOUS ADENOSINE

Extracellular adenosine may exert several physiological effects by stimulation of specific adenosine receptors. These adenosine receptors can be subdivided into A_1, A_{2A}, A_{2B} and A_3 receptors (6). By stimulation of these receptors, adenosine exerts a multitude of cardio- and vasoprotective effects by interfering with numerous mechanisms that contribute to the pathogenesis of atherosclerosis and thrombosis.

Firstly, adenosine exhibits direct and indirect effects on vascular tone. Directly, predominantly via activation of A_2 receptors, adenosine induces vasodilation (181). Indirectly, adenosine blocks the synthesis of potent vasoconstrictor factors such as angiotensin II and norepinephrine by inhibiting renin release (188). At the level of the vascular smooth muscle cell, adenosine has been reported to inhibit the release of the neurotransmitter norepinephrine and to reduce the postsynaptic vasoconstrictor response to α-adrenoceptor-stimulation in man (85, 189).
Secondly, several direct cardioprotective effects of adenosine are known. It exerts negative inotropic, chronotropic and dromotropic cardiac effects (7). Adenosine has also been reported to play a key role in ischemic preconditioning of the heart. This phenomenon concerns the observation that short periods of ischemia render the myocardium resistant to a subsequent more serious ischemic event (122). A number of studies have shown that this endogenous cardioprotective mechanism can be reduced by adenosine receptor antagonism (190), and potentiated by dipyridamole, an adenosine uptake inhibitor (191).
Thirdly, adenosine potently inhibits the aggregation of platelets (192, 193). Moreover, adenosine exerts anticoagulant activity by down-regulation of tissue factor expression on endothelial cells and by suppressing the expression of P-selectin on platelets (90, 194). These effects may well contribute to the well-known antithrombogenicity of an intact endothelial lining.
Fourthly, adenosine, at concentrations similar to those observed *in vivo*, is an important anti-inflammatory agent (92, 93). Adenosine receptor stimulation inhibits the activation of neutrophils and protects vascular endothelium from damage by neutrophils (195). Moreover, adenosine inhibits TNF-alpha production in macrophages and monocytes (196), suppresses arachidonic acid release and leukotriene biosynthesis in human neutrophils (197), and is shown to act as an endogenous activator of cellular antioxidant enzyme systems (198).
Fifthly, adenosine has been shown to play an important role in the regulation of vascular cell proliferation and death, which plays a key role in the vascular remodelling process that leads to vaso-occlusive diseases (9). Smooth muscle cell derived adenosine inhibits the proliferation and collagen synthesis of these muscle cells in an autocrine manner via adenosine A_{2B} receptor stimulation (99, 100, 199). Moreover, a selective A_2 receptor agonist was shown to reduce neointimal thickening in an animal model (200). In contrast to the growth-inhibitory effect on vascular smooth muscle cells, adenosine A_{2B} receptor stimulation induces mitogenic effects on endothelial cell (101). Besides inhibiting the growth of vascular smooth muscle cells, adenosine has been shown to induce apoptosis of human vascular smooth muscle cells via A_{2B} receptor stimulation (201). In this way, adenosine could restrict intimal hyperplasia in the early phase of atherosclerosis, but on the other hand, could also play a role in the formation of the necrotic core in advanced atherosclerosis. Regarding the effects of adenosine on vascular cell proliferation and death, the net effect would be to facilitate the recovery of blood vessels from injury by the inhibition of inappropriate migration and proliferation of vascular smooth muscle cells into the intima layer and promoting re-endothelialization via its mitogenic effects on endothelial cells (101).

All these effects make adenosine a powerful endogenous protector against arteriosclerotic and vaso-occlusive disorders and are thought to contribute to the well-documented cardioprotective properties of adenosine receptor stimulation (202, 203). In this respect, a decreased plasma and interstitial adenosine concentration in hyperhomocysteinemia could significantly contribute to the

cardiovascular complications of this disorder. The study by Chen *et al* supports this view by showing a decreased plasma and renal interstitial adenosine concentration in hyperhomocysteinemia *in vivo* (187).

A significant role for adenosine in vascular complications is not unique. Recently this was also established for hypertension in the spontaneously hypertensive rat (SHR). Extracellular adenosine levels in cultured vascular smooth muscle cells were significantly lower in SHR than in Wistar Kyoto rats (WKY) and this difference mediated the enhanced proliferation of vascular smooth muscle cells in SHR (204). These results imply that a decrease in adenosine concentration may play a role in the cardiovascular complications of hypertension, in line with the present discussion concerning the pathophysiology of hyperhomocysteinemia. To explore the situation in hyperhomocysteinemia further, the latter study could easily be replicated in CBS deficient mice, an animal model for hyperhomocysteinemia (205). Moreover, in future experiments, using microdialysis techniques, we will measure interstitial adenosine concentrations in patients with hyperhomocysteinemia and controls to evaluate the role of adenosine in the human situation.

CONCLUSION

Increased levels of homocysteine will reverse the biochemical reaction catalysed by AdoHcy hydrolase, leading to a decreased adenosine formation, or even to net extraction of adenosine from the extracellular compartment. Because of the beneficial effects of adenosine in the cardiovascular system, reduced adenosine formation during hyperhomocysteinemia may be a significant factor in the pathogenesis of the cardiovascular sequelae of this condition.

CHAPTER 3.2

ENHANCED CELLLULAR ADENOSINE UPTAKE LIMITS ADENOSINE RECEPTOR STIMULATION IN PATIENTS WITH HYPERHOMOCYSTEINEMIA

Arterioscler Thromb Vasc Biol 2005;25:109-14

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ABSTRACT

Objective – Endogenous adenosine induces several cardioprotective effects. We postulate that in patients with hyperhomocysteinemia increased intracellular formation of S-adenosylhomocysteine decreases free intracellular adenosine. Subsequently, facilitated diffusion of extracellular adenosine into cells through dipyridamole-sensitive transporters is enhanced, limiting adenosine receptor stimulation. We tested this hypothesis in patients with classical homocystinuria (n=9, plasma homocysteine 93.1 ± 24.7 μmol/l) and controls with similar baseline characteristics (n=8, homocysteine 9.1 ± 1.0).

Methods and Results - Infusion of adenosine (0.5, 1.5, 5.0 and 15.0 μg/min/dl forearm) into the brachial artery increased forearm blood flow, as measured with venous occlusion plethysmography, to 2.9 ± 0.4, 4.3 ± 0.5, 5.6 ± 1.1 and 9.6 ± 2.1 in the patients and to 2.8 ± 0.6, 4.4 ± 1.0, 9.0 ± 1.7 and 17.0 ± 3.1 ml/min/dl in controls ($P<0.05$). However, adenosine-induced vasodilation in the presence of dipyridamole (100 μg/min/dl) was similar in both groups ($P=0.9$). Additionally, in isolated erythrocytes, adenosine uptake was accelerated by incubation with homocysteine (half-time 6.4 ± 0.3 versus 8.1 ± 0.5 minutes, $P<0.001$) associated with increased intracellular formation of S-adenosylhomocysteine ($P<0.0001$).

Conclusions – In hyperhomocysteinemia, adenosine-induced vasodilation is impaired but is restored by dipyridamole. Accelerated cellular adenosine uptake probably accounts for these observations. These impaired actions of adenosine could well contribute to the cardiovascular complications of hyperhomocysteinemia.

Keywords: Adenosine, Hyperhomocysteinemia, Dipyridamole, Forearm, Nucleoside transport.

INTRODUCTION

Hyperhomocysteinemia is an independent risk factor for atherosclerosis and thromboembolism. It is poorly understood which mechanism is responsible for these cardiovascular complications.
Recently, we and others have drawn attention to a novel hypothesis, focusing on the influence of homocysteine on the metabolism of the endogenous nucleoside adenosine (187, 206). According to this hypothesis, a homocysteine-induced fall in extracellular adenosine contributes to the cardiovascular sequelae of hyperhomocysteinemia. Fundamental to this is the reversibility of the reaction in which S-adenosylhomocysteine (AdoHcy) is hydrolysed to form homocysteine and adenosine (23). Although the equilibrium constant of this reaction favors AdoHcy synthesis, under physiological conditions AdoHcy is hydrolyzed to homocysteine and adenosine, because both reaction products are rapidly metabolized. In hyperhomocysteinemia, the reaction shifts towards AdoHcy synthesis, at the expense of free intracellular adenosine. Subsequently, facilitated diffusion of extracellular adenosine into the cells through the dipyridamole-sensitive equilibrative nucleoside transporter is enhanced, limiting stimulation of membrane-bound adenosine receptors (figure 1).
By stimulation of these receptors, extracellular adenosine induces several effects, which could protect against the development of atherosclerosis and thrombosis and against ischemia-reperfusion injury (5, 206).

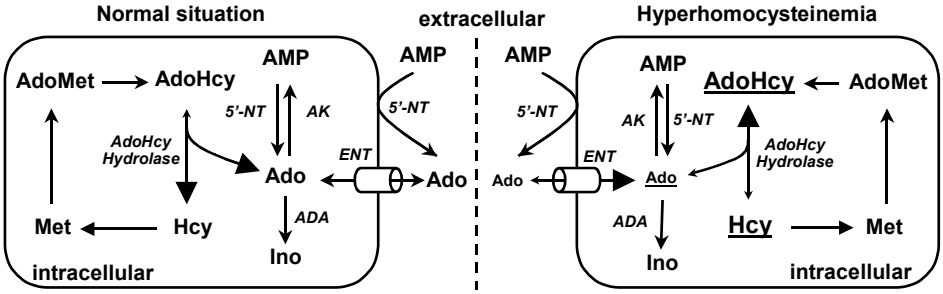

Figure 1: Simplified representation of the biochemical reactions relevant to our hypothesis. Under normal conditions, adenosine is continuously produced by the hydrolysis of AdoHcy (left). In hyperhomocysteinemia, adenosine production from AdoHcy hydrolysis is hampered or the reaction is even reversed, and AdoHcy will accumulate at the expense of free adenosine (right). Dipyridamole inhibits cellular adenosine uptake by blockade of the ENT. AMP: adenosine mono-phosphate; 5'-NT: 5'-nucleotidase; ADA: adenosine deaminase; AK: adenosine kinase; Ino: inosine; Met: methionine; ENT: equilibrative nucleoside transporter.

Particularly in situations of hypoxia or ischemia, when the concentration of adenosine rapidly increases, these effects work in concert to protect the affected tissue (207). Previous animal studies suggest that the effect of intracellular AdoHcy formation on the transmembranous adenosine concentration gradient and thus diffusion of extracellular adenosine into the cells, is most pronounced in these very situations of high concentrations of adenosine, thus limiting adenosine receptor stimulation when most needed (155, 208). Therefore, we speculate that in hyperhomocysteinemia, decreased extracellular adenosine concentrations contribute to the development of the associated cardiovascular problems.

In the present study, we addressed this issue in patients with severe hyperhomocysteinemia. We estimated basal intravascular and muscle interstitial adenosine concentration by microdialysis. Secondly, we measured adenosine-induced forearm vasodilation. According to our hypothesis, accelerated cellular adenosine uptake would decrease the amount of free extracellular adenosine able to stimulate adenosine receptors and, consequently, would attenuate adenosine-induced vasodilation in this patient group. Inhibition of cellular adenosine uptake by dipyridamole should restore this diminished response. Additionally, we aimed to more directly demonstrate homocysteine-induced increased intracellular formation of AdoHcy and subsequent accelerated adenosine uptake in isolated human erythrocytes.

METHODS

Subjects

After approval of the local ethics committee, adult patients with hyperhomocysteinemia due to homozygous cystathionine beta-synthase deficiency from our outpatient clinic were asked to participate if their fasting total plasma homocysteine was more than 20 μmol/l, despite treatment. Exclusion criteria were mental retardation, previous vascular events, asthma and oral anticoagulation. Twelve patients were considered eligible, and nine agreed to participate and signed informed consent. Their homocysteine-lowering therapy consisted of vitamin B6 (250-750 mg, n=9), folic acid (5 mg, n=9), vitamin B12 (10 μg orally each day or 1 mg intramuscularly each two months, n=7) and betaine anhydricum (6 g, n=7). Further treatment consisted only of acetylsalicylic acid 80 mg in one patient (stopped one week before the experiment) and alendronic acid 70 mg weekly in 2 patients. A control group of eight healthy volunteers was composed, with similar age and BMI as the patient group.

Instrumentation

All tests were performed in the morning in a temperature-controlled laboratory (23°C), after an overnight fast and 24 hours of caffeine abstinence. After local anesthesia (xylocaine 2%), a microdialysis probe (CMA/70 microdialysis catheter, Microdialysis AB, Stockholm, Sweden) was inserted into the flexor digitorum superficialis muscle of the non-dominant arm, guided by a 14-gauge venflon cannula. An identical microdialysis probe was inserted into a deep antecubital vein of the same arm. Each probe was connected to a microdialysis pump (CMA/107 microdialysis pump, Microdialysis AB, Stockholm, Sweden), and continuously perfused with isotonic saline at 2 μl/min. The effluent was collected at 15-minute intervals to obtain 30 μl samples (dialysate). Samples were stored at –20 °C until analysis.

Subsequently, the brachial artery of the non-dominant arm was cannulated and forearm blood flow (FBF) was measured in each arm using mercury-in-silastic venous occlusion plethysmography as described previously (141). Each drug dosage was infused for 5 minutes.

In the first hour of the study, blood was drawn for determination of plasma total homocysteine, AdoHcy, S-adenosylmethionine (AdoMet), vitamin B6, folate, vitamin B12 and cholesterol.

Experimental protocol

Immediately after insertion of the microdialysis probes, dialysate sampling was started. Two hours after insertion, both microdialysis probes were removed and placed in isotonic saline for *in vitro* calibration, as previously described (209).

Subsequently, baseline FBF was measured during infusion of saline followed by infusion of increasing dosages of adenosine (0.5, 1.5, 5 and 15 μg/min/dl forearm). After 30-minutes of equilibration, baseline FBF measurement was repeated, followed by infusion of dipyridamole (100 μg/min/dl) and increasing dosages of adenosine (0.15, 0.5 and 1.5 μg/min/dl) on top of dipyridamole infusion. In combination with dipyridamole, we used lower concentrations of adenosine because of the well-known potentiating effect of dipyridamole on adenosine-induced vasodilation (154). Finally, maximal vasodilation was measured during post-occlusive reactive hyperemia to test for possible structural vascular changes in the patient group, as described previously (141).

Adenosine uptake in isolated erythrocytes

In 6 additional healthy volunteers, erythrocytes were isolated for *in vitro* experiments. In hyperhomocysteinemia, erythrocytes are relevant in the regulation of circulating endogenous adenosine because adenosine is efficiently taken up by erythrocytes via the dipyridamole-sensitive transporter and because homocysteine and AdoHcy are increased in erythrocytes of patients with hyperhomocysteinemia (210, 211)-(212).

Freshly isolated erythrocytes were resuspended in MOPS-buffer to obtain a 2% solution. 50 μl portions were incubated at 37°C with L-homocysteine (100 μM) in DTT and with DTT alone for 10 minutes (paired experiments). Subsequently, adenosine was added in a final concentration of 1 μM. After 0, 3, 6, 10 and 15 minutes, adenosine uptake and deamination was completely blocked with high dose dipyridamole (10 μM) and erythro-9-(2-hydroxynon-3-yl)-adenine (8 μM), respectively. Subsequently, after centrifugation through a dibutylphtalate layer, the adenosine concentration in the supernatant and the AdoHcy concentration in the erythrocytes was determined. The effect of homocysteine on adenosine uptake was maximal after 6 minutes of uptake. In order to investigate the adenosine concentration dependency of this homocysteine effect, an additional series of experiments was conducted with 6 minutes of adenosine uptake, but with a variable adenosine concentration ranging from 0.125 μM to 2 μM (n=4). Finally, we determined the inhibiting effect of dipyridamole (0.2 μM, 5 minutes incubation) on the accelerating effect of homocysteine with 6 minutes of adenosine uptake (n=2).

Drugs and solutions

Solutions of adenosine (Adenocor, Sanofi-Synthelabo, Maassluis, the Netherlands) and dipyridamole (Persantin, Boehringer Ingelheim, Espana S.A., Spain) were freshly prepared with NaCl 0.9% as solvent. L-homocysteine was freshly prepared immediately before each *in vitro* experiment from L-homocysteine thiolactone as previously described (168).

Analytical procedures

Dialysate adenosine concentration was determined by HPLC with reversed-phase ion-pairing separation and UV-detection. Plasma homocysteine was determined by RP-HPLC as described

previously (213). Plasma AdoHcy and AdoMet was determined by tandem mass-spectrometry, based on the work of Struys *et al* (214).

Statistics

Values are expressed as mean ± standard error (SE), unless otherwise stated. $P<0.05$ is considered statistically significant. Since plasma concentrations of homocysteine, AdoHcy, AdoMet and vitamins did not show a Gaussian distribution ($P>0.1$; Shapiro-Wilk test for normality), the Mann-Whitney test was used to compare groups. Other baseline parameters were normally distributed, and consequently a Student's t-test was used.

To compare adenosine-induced vasodilation between the two groups, a repeated measures ANOVA was used. Finally, for each subject, the area under the curves (AUC) of change in FBF was calculated for the adenosine-induced vasodilation with and without dipyridamole. The ratio of the AUC (with dipyridamole) and the AUC (without dipyridamole) was used to quantify the effect of dipyridamole. Since these ratios were not normally distributed, a Mann-Whitney test was used to compare groups.

In the *in vitro* experiments, the decrease of extracellular adenosine in time was fitted according to one phase exponential decay (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA) and half times were compared with paired Student's t-test.

RESULTS

Baseline characteristics are shown in table 1.

Table 1: baseline characteristics. *Intra-arterially and †electrocardiographically measured during saline infusion. ‡$P<0.005$ compared with control group. §Upper limit of detection 270 nmol/l.

	Patients	Controls
Number	9	8
Sex (male/female)	8/1	6/2
Age (years)	36.3±2.2	36.8±4.9
BMI (kg/m^2)	25.4±0.9	24.4±1.5
Systolic blood pressure (mmHg)*	126±4	116±3
Diastolic blood pressure (mmHg)*	72±4	67±3
Heart rate (bpm)†	64±4	57±2
Glucose (mmol/l)	4.9±0.1	5.2±0.3
Cholesterol (mmol/l)	4.9±0.3	4.6±0.3
Total homocysteine (µmol/l)	93.1±24.7‡	9.1±1.0
S-adenosylhomocysteine (nmol/l)	41.8±10.0‡	7.7±0.8
S-adenosylmethionine (nmol/l)	389.8±97.1‡	96.8±8.5
Vitamin B12 (pmol/l)	258±18	284±30
Vitamin B6 (nmol/l)	4315±305‡	75±5
Folate (nmol/l)§	155±39‡	12±1

Plasma concentrations of total homocysteine, AdoHcy and AdoMet were higher in the patient group (P<0.005). Treatment with high dose folic acid and pyridoxine resulted in higher plasma concentrations of folate and vitamin B6 in the patient group (P<0.005). In one patient, we were not able to insert the intravenous probe and intra-arterial cannula, and consequently only data on interstitial adenosine were available.

Microdialysis experiments

Immediately after intramuscular insertion, dialysate adenosine concentration is known to be high due to myocyte damage and decreases to baseline level within one hour (209). To estimate basal interstitial adenosine concentration, we averaged the dialysate concentration of the two consecutive dialysate samples taken 1.5 hours after insertion. In the patient group basal adenosine concentration was 96 ± 21 nmol/l (n=7; 2 patients were excluded because of extremely low concentration of creatine and phosphocreatine in the first microdialysis sample, indicating misplacement of the probe), in the control group 73 ± 9 nmol/l (n=8, P=0.3). The dialysate adenosine concentration from the intravascular probe was at steady-state immediately after insertion. Therefore, we averaged the values of all dialysate samples to one value. In the patient group this baseline concentration yielded 142 ± 33 nmol/l (n=8), in the control group 135 ± 25 nmol/l (n=8, P=0.7). Adenosine recovery of the intramuscular and intravascular probes was 48 ± 4 % and 47 ± 5 % for patients and 47 ± 4 % and 39 ± 2 % for controls, respectively (P=0.9 and 0.2, respectively).

Plethysmography experiments

Baseline FBF in the infused arm was 2.0 ± 0.2 and 2.5 ± 0.5 ml/min/dl for patients and controls, respectively (n=8; P=0.4). During infusion of increasing adenosine dosages, FBF in the patient group was 2.9 ± 0.4, 4.3 ± 0.5, 5.6 ± 1.1 and 9.6 ± 2.1 ml/min/dl, respectively (figure 2). In the control group, FBF was 2.8 ± 0.6, 4.4 ± 1.0, 9.0 ± 1.7 and 17.0 ± 3.1 ml/min/dl, respectively. This adenosine-induced vasodilation was attenuated in the patient group (P<0.05).

After 30-minute equilibration, baseline FBF was 2.7 ± 0.3 and 3.1 ± 0.7 ml/min/dl in the patient group and control group, respectively (n=8; P=0.6). Infusion of dipyridamole increased FBF to 4.3 ± 0.5 ml/min/dl in patients and to 4.7 ± 1.0 in controls (P=0.7 between groups). Subsequent infusion of adenosine on top of dipyridamole increased FBF to 10.9 ± 1.6, 16.3 ± 2.6 and 27.2 ± 2.9 ml/min/dl in patients and to 8.8 ± 1.4, 14.8 ± 2.4 and 24.4 ± 4.0 in controls, respectively (P=0.9). The ratio of the AUC for adenosine-induced vasodilation with dipyridamole and without dipyridamole was 0.40 ± 0.08 in the patient group and 0.16 ± 0.03 in the control group (P<0.05). Moreover, with both experimental groups taken together, there was a negative correlation between total plasma homocysteine concentration and the AUC for adenosine-induced vasodilation (Spearman r=-0.53, P=0.035) and a positive correlation between plasma homocysteine and the effect of dipyridamole expressed as the ratio of the AUC's for adenosine-induced vasodilation with and without concomitant infusion of dipyridamole (Spearman r=0.59, P=0.015).

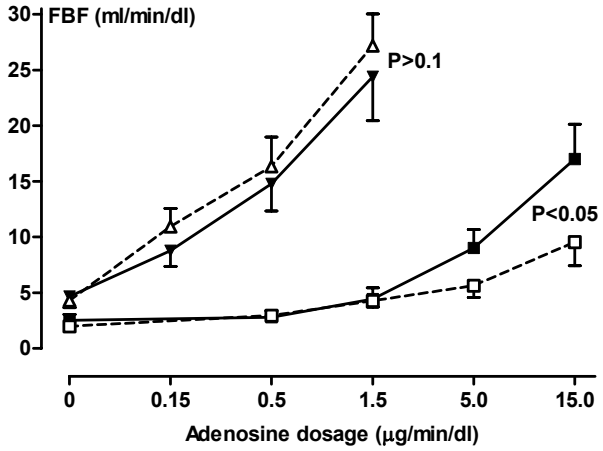

Figure 2: Adenosine-induced vasodilation without dipyridamole (squares) and during co-infusion with dipyridamole (triangles) in patients (open symbols; mean ± SE, n=8) and controls (filled symbols; n=8).

During the experiment, neither mean arterial pressure (MAP), nor the FBF in the control arm differed between the patient group and the control group (P>0.1; ANOVA for repeated measures). The effects of adenosine infusion on heart rate and blood pressure are shown in table 2.

Table 2: Systemic effects of adenosine infusion. *Significant increase in heart rate as compared to baseline 1 (P<0.05) and †baseline 2 (P<0.001). No differences between groups.

	Heart rate		Mean arterial pressure	
	Patients	Controls	Patients	Controls
baseline 1	62±4	57±2	93±4	86±3
ado 0.5	61±4	55±1	93±4	86±3
ado 1.5	63±4	56±1	93±4	87±4
ado 5.0	62±4	56±1	96±4	83±4
ado 15.0	65±3*	57±2	96±4	87±3
baseline 2	64±4	57±2	96±4	89±3
dipy	66±5	57±2	97±4	89±3
dipy+ado 0.15	69±4	60±2	97±4	88±3
dipy+ado 0.5	70±4	63±2	99±4	90±4
dipy+ado 1.5	76±5†	70±2†	99±5	89±4

Concomitant infusion of adenosine with dipyridamole was associated with an increase in heart rate in both groups (ANOVA for repeated measures, P<0.001). Also, infusion of adenosine without dipyridamole was associated with an increase in heart rate in the patient group (P<0.05). Blood pressure was not influenced by adenosine infusion. There were no differences between both groups.

Finally, minimal forearm vascular resistance (MAP/FBF) during post-occlusive reactive hyperemia was 2.1±0.2 and 2.4±0.2 AU for patients and controls, respectively (P=0.3).

Adenosine uptake in isolated erythrocytes

Plasma homocysteine of the six subjects in this study averaged 9.1±0.9 μM. Adenosine uptake into the erythrocytes and subsequent metabolism results in a decrease of extracellular adenosine in time (figure 3a). Adenosine uptake was accelerated by incubation with homocysteine (half time 6.4±0.3 versus 8.1±0.5 minutes, P<0.001). Intracellularly, AdoHcy increased in time in cells incubated with homocysteine, but not in control samples (Figure 3b, P<0.0001, ANOVA for repeated measures). At 6 minutes, adenosine in the supernatant was lower in the homocysteine-incubated cells. This absolute homocysteine-induced difference in adenosine concentration significantly increased when the initial adenosine concentration was varied from 0.125 μM to 2.0 μM (figure 3c and 3d, P<0.05, ANOVA for repeated measures).

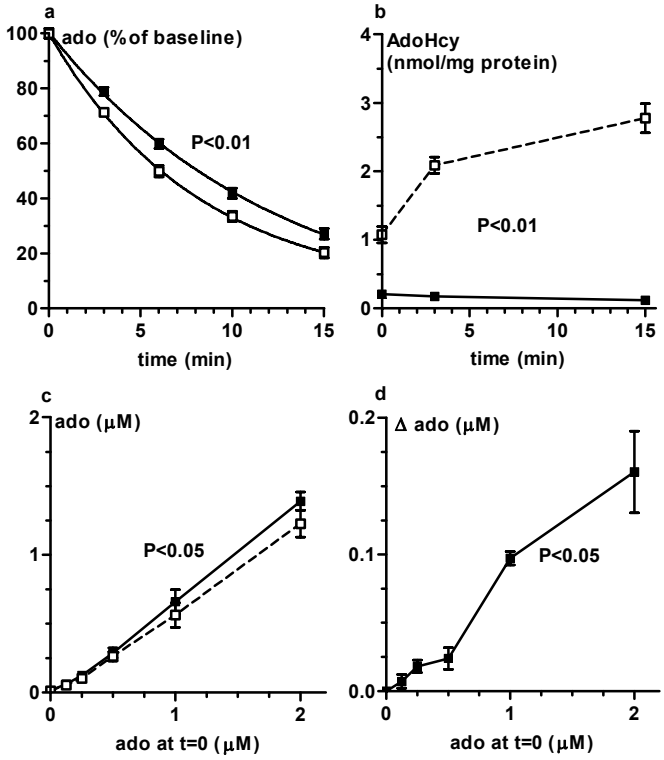

Figure 3: Time-course of adenosine concentration in the supernatant (a) and the intracellular AdoHcy concentration (b) after addition of 1 μM of adenosine to erythrocytes. Also shown is the adenosine concentration in the supernatant 6 minutes after the addition of variable concentrations of adenosine (c; n=4) and the absolute homocysteine-induced difference in adenosine concentration after 6 minutes of uptake (d; n=4)(open squares = incubation with homocysteine; filled squares = control group).

Finally, dipyridamole significantly inhibited the accelerating effect of homocysteine on cellular adenosine uptake. Homocysteine decreased the free extracellular adenosine concentration after 6 minutes of adenosine uptake with 14.5±0.5% in the absence and with 2.9±0.5% in the presence of dipyridamole (Figure 4, P<0.05, n=2).

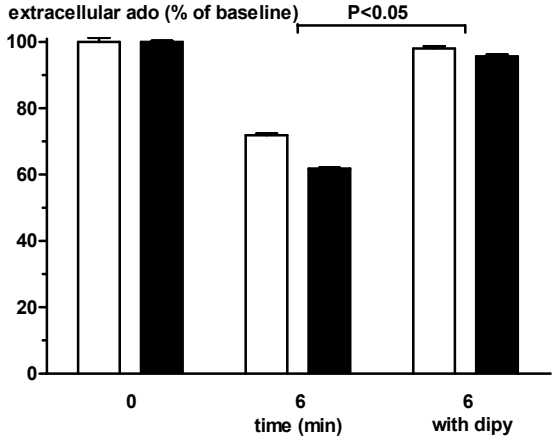

Figure 4: Extracellular concentration of adenosine after 0 and 6 minutes of adenosine uptake by isolated human erythrocytes in the absence (open bars) and presence of homocysteine (filled bars). Dipyridamole significantly reduces the accelerating effect of homocysteine on adenosine uptake (P<0.05, n=2).

DISCUSSION

In the present study, we show for the first time that adenosine-induced forearm vasodilation is attenuated in patients with hyperhomocysteinemia due to classical homocystinuria and that this is completely restored by co-infusion of the adenosine uptake inhibitor dipyridamole.

The association between hyperhomocysteinemia and atherosclerosis and thrombosis was first established in patients with classical homocystinuria (163). Without treatment, 50% of suchlike patients suffer a vascular event before the age of 30 years (160). Treatment is aimed at minimizing the biochemical abnormalities and consists of pyridoxine (vitamin B6) and folic acid and, if necessary, betaine anhydricum and vitamin B12 (162). This treatment regimen significantly improves vascular outcome (215). Our patients continued to have elevated plasma homocysteine levels, despite treatment. Due to the defective degradation of homocysteine to cystathionine, homocysteine can only be remethylated to methionine or converted to AdoHcy. This altered metabolic profile is illustrated by the increased plasma concentrations of AdoHcy and AdoMet in our patient group. Previous *in vivo* experiments on vascular function in this patient group are scarce and demonstrated impaired flow-mediated dilation and carotid artery wall hypertrophy (216-218).

In the present study, adenosine-induced forearm vasodilation was reduced in patients with hyperhomocysteinemia. Based on the observations that dipyridamole restores this impaired vasodilation and that in erythrocytes homocysteine accelerates cellular adenosine uptake, we conclude that this impaired adenosine-induced vasodilation is caused by accelerated cellular adenosine uptake. Adenosine-induced vasodilation is partly endothelium-dependent (76). We did not use alternative endothelium-dependent vasodilators to test for endothelial function in our patient group. However, as shown by previous studies in patients with classical homocystinuria, endothelial dysfunction could well also be present in our patients. Could endothelial dysfunction or structural vascular damage, both previously described in a comparable patient group, challenge our conclusion? In our opinion this is not the case. The observation that dipyridamole restored adenosine-induced vasodilation suggests that accelerated adenosine uptake via the equilibrative nucleoside transporter, rather than endothelial dysfunction accounts for the attenuated vasodilation. It needs to be realized that dipyridamole is proposed to have alternative mechanisms of action besides inhibition of nucleoside transport (103). Phosphodiesterase inhibition could theoretically potentiate endothelium-dependent cGMP-mediated dilation, thus improving this portion of adenosine-induced vasodilation. However, in an identical experimental model as in the present study, our group has previously shown that dipyridamole-induced local vascular effects are indeed solely due to adenosine uptake inhibition. Dipyridamole potentiated the vasodilator response to adenosine (154) and dipyridamole-induced vasodilation (100 μg/min/dl) was inhibited by the adenosine receptor antagonist theophylline (106). Moreover, we showed in isolated erythrocytes that homocysteine indeed accelerates cellular adenosine uptake, which is counteracted by dipyridamole. Although in this model the effects of homocysteine are rather modest, these *in vitro* observations provide additional evidence that accelerated cellular adenosine uptake might account for the observed impaired adenosine-induced vasodilation.
Minimal forearm vascular resistance was similar in both groups. This parameter accurately reflects structural arteriolar status (143), indicating that in our patient group, at least in the forearm vascular bed, no structural vascular changes were present. It should be mentioned that reactive hyperemia is shown to be partially adenosine dependent (72). This portion of the hyperemia in our study would theoretically be diminished in the patients with hyperhomocysteinemia. However, because of the maximal stimulus for vasodilation after 13 minutes of ischemia, other mediators probably compensate for this decrease in adenosine-induced vasodilation (143).

In previous experiments, it was shown in guinea pig hearts (183, 184) and rat brain (182) that perfusion with homocysteine (thiolactone) decreased organ release of adenosine. The results from our *in vitro* experiments showed that also in human erythrocytes, homocysteine accelerates cellular uptake of adenosine. Moreover, we demonstrated that this is indeed associated with increased intracellular formation of AdoHcy. Finally, this effect of homocysteine is more pronounced at higher concentration of adenosine (figure 2 and 3). This observation could well explain why baseline endogenous adenosine, as estimated by microdialysis, was not reduced in our patient group. This is in strong contrast to the study by Chen *et al*, which demonstrated an approximately 50% reduction in baseline endogenous adenosine concentration induced by mild elevation of plasma homocysteine from 6.7±0.4 to 14.7±0.5 μmol/l (187). This discrepancy could be caused by the differences between man and rat concerning protein-binding of homocysteine (208), AdoHcy

hydrolase activity (23), and characteristics of the equilibrative nucleoside transporter (219). Also, the experimental methionine-induced hyperhomocysteinemia in the rat model differs from the hyperhomocysteinemia in our patient group, possibly affecting tissue and cellular distribution of homocysteine. But most importantly, the results from our *in vitro* studies suggest that the effect of AdoHcy synthesis on the rate of cellular adenosine uptake is limited to situations of high extracellular adenosine concentrations, as for example in ischemia, when myocardial interstitial adenosine concentration can increase up to 40-fold in pigs (155). A recent microdialysis study on the pig heart showed that local application of homocysteine reduced dialysate adenosine concentration during hypoxia, but not during normoxia (155). Likewise, Kloor *et al* concluded from a rat study that homocysteine is the rate limiting factor for AdoHcy synthesis in hypoxic conditions, when tissue levels of adenosine are elevated, whereas in normoxia the availability of free adenosine is rate limiting (208). This suggests that also *in vivo*, the effect of homocysteine on adenosine concentration is more pronounced, and therefore more easily detected, in situations of high concentrations of adenosine, than under baseline conditions. Unfortunately, we were not able to collect microdialysis samples during adenosine infusion or during ischemia.

We have shown that the vasodilating effect of adenosine, which was reduced in hyperhomocysteinemia, was completely restored by the nucleoside uptake inhibitor dipyridamole. Extrapolating this finding, dipyridamole would be beneficial in patients with hyperhomocysteinemia, by preserving the protective cardiovascular effects of adenosine in hypoxia or ischemia. To our best knowledge, dipyridamole has never been tested systematically in these patients in clinical trials.

Several limitations of the present study need to be discussed. First, we used a group of patients with classical homocystinuria, using high doses of vitamins, including vitamin B6 and folic acid. It remains to be established whether changes in adenosine metabolism are also important in patients with mild hyperhomocysteinemia. Considering the vitamin therapy in our patient group, we are not aware of any actions of these vitamins on nucleoside transport. Secondly, considering the large interindividual variation, microdialysis may lack sufficient sensitivity to exclude subtle differences in baseline adenosine concentration between the two study groups. Moreover, the values obtained with microdialysis might not reflect the adenosine concentrations in specific microenvironments, such as near the endothelial lining.

In conclusion, in patients with severe hyperhomocysteinemia, adenosine-induced effects are impaired, which could contribute to the cardiovascular complications of this disease.

ACKNOWLEDGEMENTS

Financial support by the Netherlands Organization for Scientific Research (NPR), the Royal Netherlands Academy of Arts and Sciences (GAR), the Netherlands Heart Foundation (HJB), and the European Union (project number: QLK1-CT-2000-00069) is gratefully acknowledged.

The authors thank D. Oppenraaij for technical assistance in the determinations of plasma homocysteine, AdoHcy and AdoMet and E. van Balen for her contribution to the *in vitro* experiments.

CHAPTER 3.3

REDUCED ADENOSINE RECEPTOR STIMULATION AS A PATHOGENIC FACTOR IN HYPERHOMOCYSTEINEMIA

Clin Chem Lab Med 2005;43:1001-6

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ABSTRACT

In this review we discuss the hypothesis and current evidence that a decreased concentration of the endogenous purine-nucleoside adenosine contributes to the increased cardiovascular risk of patients with hyperhomocysteinemia. In hyperhomocysteinemia the reaction equilibrium of the reaction catalyzed by S-adenosylhomocysteine hydrolase will shift towards synthesis of S-adenosylhomocysteine, at the expense of free adenosine. Adenosine receptor stimulation induces several cardiovascular protective effects, such as vasodilation, inhibition of thrombocyte aggregation, of inflammation and of vascular smooth muscle cell proliferation. A decreased adenosine concentration could therefore well contribute to the cardiovascular complications of hyperhomocysteinemia. Previous animal studies have shown that administration of homocysteine decreases extracellular adenosine, associated with increased synthesis of S-adenosylhomocysteine. Recently, we showed that in patients with classical homocystinuria, cellular adenosine uptake is enhanced, thus limiting adenosine-induced vasodilation. These observations provide us with additional pharmacological targets, such as adenosine uptake inhibition, to reduce cardiovascular risk in patients with hyperhomocysteinemia.

Keywords: adenosine; cardiovascular disease; dipyridamole; hyperhomocysteinemia.

INTRODUCTION

Hyperhomocysteinemia is an independent risk factor for atherosclerosis and thrombosis (161). McCully was the first to link elevated plasma homocysteine concentration with vascular disease in patients with homocystinuria (163). In classical homocystinuria, homozygous deficiency of the cystathionine beta-synthase enzyme (CBS) results in severe hyperhomocysteinemia (162). Without treatment, half of all patients with this metabolic defect will suffer a vascular event before the age of thirty (160). Later, the association between plasma homocysteine concentration and cardiovascular events was also established for mild hyperhomocysteinemia (161). Also, elevated plasma homocysteine is associated with increased mortality due to myocardial infarction (220) and in patients without prior myocardial infarction an increased plasma homocysteine concentration is associated with an increased incidence of congestive heart failure (221).

Although epidemiological evidence for the association of hyperhomocysteinemia and cardiovascular morbidity and mortality is convincing, the pathophysiological mechanism mediating this association has not yet been elucidated. Recently, we and others have proposed that in hyperhomocysteinemia, the concentration of the endogenous nucleoside adenosine is decreased and that this contributes to the increased cardiovascular risk (159, 187, 206).

In the present review, we will discuss the potential role of adenosine in the pathophysiology of the cardiovascular complications of hyperhomocysteinemia and the experimental evidence, both *in vitro* and *in vivo* in humans, supporting this mechanism.

PATHOPHYSIOLOGICAL MECHANISMS FOCUSING ON HOMOCYSTEINE

Although many studies have been conducted to unravel why patients with hyperhomocysteinemia are predisposed to cardiovascular events, up to now no coherent, generally accepted view has emerged. According to most studies, endothelial dysfunction and injury are key components in the development of atherosclerosis and thrombosis (162). *In vivo*, experimental hyperhomocysteinemia rapidly impairs endothelial function (165, 166). Additionally, *in vitro*, deleterious effects of homocysteine have been described not only on endothelial cells, but also on platelets, vascular smooth muscle cells, coagulation and inflammation (162). There is evidence that homocysteine exerts these effects by promoting oxidative damage, inflammation, reducing NO-bioavailability, and by direct promitogenic effects on vascular smooth muscle cells (222, 223). However, many *in vitro* studies suffer from methodological shortcomings, especially due to the use of supraphysiological concentrations of homocysteine (169).

Recently, several studies suggested that not homocysteine itself, but rather one of its metabolic precursors, S-adenosylhomocysteine (AdoHcy), is responsible for the adverse cardiovascular effects of hyperhomocysteinemia (170-172). When the intracellular concentration of homocysteine increases, the equilibrium of the reaction catalysed by AdoHcy hydrolase shifts towards AdoHcy synthesis from homocysteine and adenosine and, in turn, AdoHcy potently inhibits transmethylation reactions (23). Interestingly, a recent case-control study showed that plasma AdoHcy concentration was a better predictor of cardiovascular disease than plasma homocysteine (175).

In hyperhomocysteinemia, the shift of the reaction equilibrium of the reaction catalysed by AdoHcy hydrolase not only increases intracellular AdoHcy but also inevitably lowers intracellular free adenosine, which will be highlighted in the next section.

MODULATION OF ADENOSINE METABOLISM IN HYPERHOMOCYSTEINEMIA

In every cell type, homocysteine is formed exclusively by the hydrolysis of AdoHcy. Hyperhomocysteinemia is caused by impaired degradation of homocysteine, either by inherited enzymatic defects or by vitamin deficiencies. It is essential to realize that the activity of the enzymes involved in the metabolism of homocysteine are highly tissue-specific (224, 225). Irreversible degradation to cystathionine occurs predominantly in the liver, whereas outside liver and kidney, homocysteine metabolism is solely dependent on folate mediated remethylation and cellular efflux (225). This is illustrated by van der Molen *et al*, who showed that in cultured endothelial cells from a patient with CBS deficiency, homocysteine efflux was not increased, even in the presence of high concentrations of methionine (226). In patients with CBS deficiency, high plasma concentrations of homocysteine and methionine thus result from impaired metabolism in hepatocytes. Cells in the cardiovascular system, such as endothelial cells, vascular smooth muscle cells, and cardiomyocytes are subsequently exposed to these high concentrations of methionine and homocysteine.

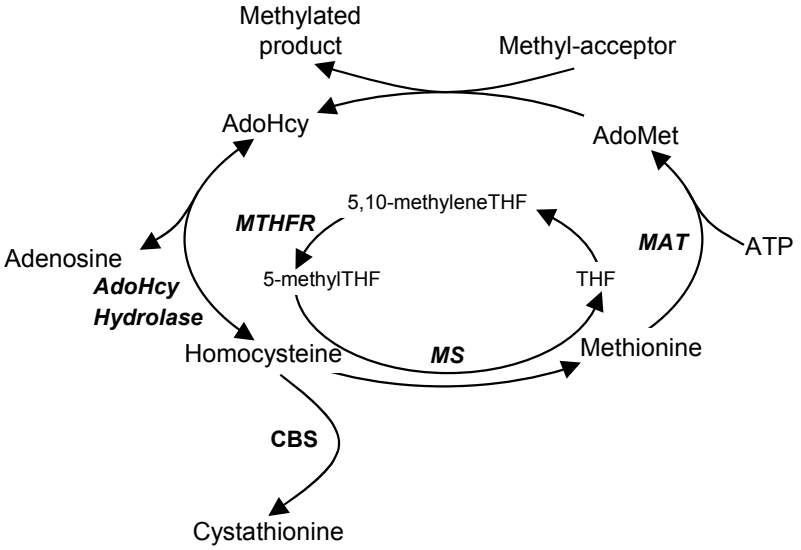

Figure 1: Schematic representation of the intracellular metabolism of homocysteine in the liver. MAT: methionine adenosyl transferase; MS: methionine synthase; MTHFR: methylenetetrahydrofolate reductase; THF: tetrahydrofolate.

In the metabolism of homocysteine, only the reaction catalysed by AdoHcy hydrolase is able to function in both directions (figure 1) (23). The equilibrium constant of this reaction strongly favours AdoHcy synthesis. Nonetheless, the reaction is driven in the opposite direction, because under normal conditions both reaction products are rapidly removed from the cytosol (23). The K_m of this enzyme for homocysteine has been reported to be 80-160 μM (179, 208). As the isoform of the methionine adenosyl transferase enzyme which is present outside the liver seems to operate near

its maximal capacity already at normal tissue concentrations of methionine, additional production of homocysteine from increased methionine is prevented (227). Consequently, an increased homocysteine concentration in these cardiovascular cells will shift the reaction equilibrium of the abovementioned reaction in the direction of AdoHcy synthesis, either by inhibition of AdoHcy hydrolysis or by increased AdoHcy synthesis, causing AdoHcy to accumulate at the expense of free intracellular adenosine (figure 2) (206). In this situation, intracellular formation of adenosine is hampered or free adenosine is even extracted from the cytosol, thus increasing the transmembranous concentration gradient for adenosine, resulting in accelerated cellular uptake of adenosine through the dipyridamole sensitive equilibrative nucleoside transporter (figure 2).

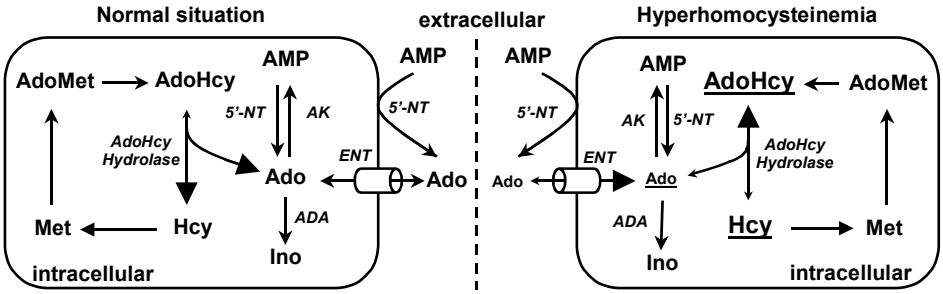

Figure 2: Simplified representation of the biochemical reactions relevant to our hypothesis. Under normal conditions, adenosine is continuously produced by the hydrolysis of AdoHcy (left). In hyperhomocysteinemia, adenosine production from AdoHcy hydrolysis is hampered or the reaction is even reversed, and AdoHcy will accumulate at the expense of free adenosine (right). ADA: adenosine deaminase; AK: adenosine kinase; AMP: adenosine mono-phosphate; ENT: equilibrative nucleoside transporter; Ino: inosine; MAT: methionine adenosyl transferase; Met: methionine; 5'-NT: 5'-nucleotidase. Adapted with permission from Riksen *et al*. Arterioscler Thromb Vasc Biol 2005;25:109-114.

Under normoxic conditions, adenosine is produced continuously in several cell types, such as endothelial cells and vascular smooth muscle cells, originating mainly from intra- and extracellular hydrolysis of adenosine mono-phosphate (AMP) by endo- and ecto-5'-nucleotidase (22). A second pathway in adenosine formation is the continuous hydrolysis of AdoHcy (179). Under hypoxic conditions, the hydrolysis of AMP greatly increases and predominates the other pathway. Extracellular adenosine is quickly taken up by neighbouring cells by nucleoside transporters and is degraded to inosine by adenosine deaminase or rephosphorylated by adenosine kinase. As such, plasma and interstitial concentrations of adenosine are determined by the sum of these processes, and an increased intracellular formation of AdoHcy in hyperhomocysteinemia should result in a decreased intra- and extracellular adenosine concentration, provided that other routes of adenosine metabolism are unchanged.

CARDIOVASCULAR EFFECTS OF ENDOGENOUS ADENOSINE

To adequately appreciate the deleterious effects which could result from a decrease in the extracellular concentration of adenosine, it is essential to briefly review the effects of adenosine

receptor stimulation (figure 3). Endogenous adenosine has since long been characterized as "retaliatory metabolite", describing the hypothesis that adenosine is released in response to a wide variety of stressful and potentially injurious stimuli and subsequently, in an autocrine fashion, protects organ systems against these stimuli (10).

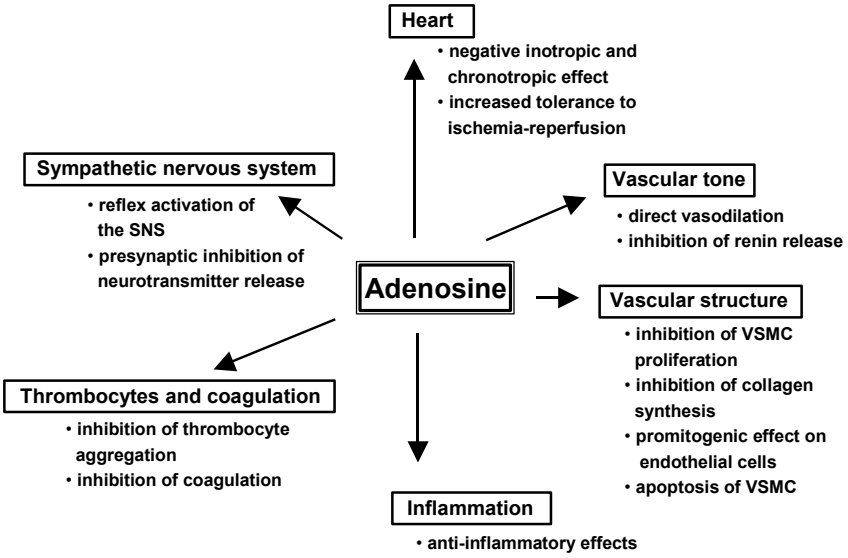

Figure 3: Overview of the most important cardiovascular effects of adenosine receptor stimulation. SNS: sympathetic nervous system; VSMC: vascular smooth muscle cell.

Adenosine receptors are membrane-bound G-protein coupled receptors and can be subdivided into A_1, A_{2A}, A_{2B} and A_3 subtypes (6). On the heart, adenosine exerts direct negative inotropic, dromotropic and chronotropic effects (7). In addition, adenosine, released during brief periods of ischemia, renders the heart more resistant to a subsequent more prolonged period of ischemia, a phenomenon called "ischemic preconditioning" (228). Considering vascular tone, adenosine is a potent vasodilator, both directly by stimulation of endothelial and vascular smooth muscle cells, and indirectly by blocking the synthesis of vasoconstrictors such as angiotensin II by inhibiting renin release (181, 188). Also, at the level of the vascular smooth muscle cell, adenosine presynaptically inhibits the release of norepinephrine and reduces the postsynaptic vasoconstrictor response to alpha-adrenoceptor stimulation (85, 189). By inhibiting aggregation of thrombocytes and expression of P-selectin and tissue factor, adenosine receptor stimulation prevents the formation of thromboemboli (90, 192, 194). Furthermore, at concentrations similar to those observed *in vivo*, adenosine is an important anti-inflammatory agent. Increased endothelial barrier function, decreased endothelial adhesion molecule expression, suppression of superoxide generation by stimulated neutrophils and modulation of cytokine production all work in concert to limit inflammatory injury (10). Finally, adenosine receptor stimulation importantly regulates

vascular cell proliferation and death, which plays a key role in the vascular remodelling process that ultimately leads to vaso-occlusive disease and heart failure (9). Smooth-muscle-cell-derived adenosine inhibits proliferation and collagen synthesis of these muscle cells in an autocrine fashion and promotes apoptosis (9, 201). In contrast to the growth-inhibitory effects on vascular smooth muscle cells, adenosine receptor stimulation induces mitogenic effects on endothelial cells (101). Regarding the effects of adenosine on vascular cell proliferation and death, the net effect would be to facilitate the recovery of blood vessels from injury by the inhibition of inappropriate migration and proliferation of vascular smooth muscle cells into the intima layer and promoting re-endothelialization via its mitogenic effects on endothelial cells (101). Particularly in situation of hypoxia, ischemia or inflammation, when the extracellular concentration of adenosine rapidly increases, all these effects work in concert to protect the affected tissue (207).

In hyperhomocysteinemia, extracellular adenosine concentration will fall. When integrating the aforementioned effects of adenosine, it can easily be appreciated that reduced adenosine receptor stimulation will promote a phenotype predisposed to cardiovascular morbidity and mortality. Many of the adverse cardiovascular effects of hyperhomocysteinemia, such as impaired vasodilation, endothelial dysfunction, vascular smooth muscle cell proliferation, increased collagen synthesis, platelet activation, thrombogenesis, and inflammation, fit well into the expected phenotype of impaired adenosine receptor stimulation.

EVIDENCE FOR REDUCED ADENOSINE CONCENTRATION IN HYPERHOMOCYSTEINEMIA

More than twenty years ago, it was already demonstrated in animal studies that homocysteine is able to modulate metabolism of endogenous adenosine (183). In isolated perfused guinea pig and rat hearts the addition of L-homocysteine thiolactone to the perfusate reduced the concentration of endogenous adenosine in the coronary effluent (179, 183, 184). Later it was shown *in vivo* in the rat brain using microdialysis that administration of L-homocysteine thiolactone (in a concentration ranging from 10 μmol/l to 1 mmol/l) decreased dialysate adenosine concentration and significantly attenuated ischemia-induced increased adenosine release (182). In isolated guinea pig, canine and rat hearts and in rat kidney and brain, the addition of homocysteine (thiolactone) significantly increased tissue concentration of AdoHcy (179, 183, 208, 229, 230).

To appreciate how the magnitude of the effect of homocysteine on adenosine kinetics is dependent on extracellular adenosine concentration, it is important to differentiate between normoxic and hypoxic/ischemic situations. The effect of homocysteine on the tissue concentration of AdoHcy and the extracellular adenosine concentration is most pronounced in situations of ischemia or hypoxia, when extracellular adenosine concentration are known to be particularly high (183, 184, 208, 230). Thus it is likely that the availability of free adenosine is rate limiting for AdoHcy synthesis in normoxia, whereas only in hypoxia or ischemia, the homocysteine concentration is rate limiting (208).

Although these studies convincingly illustrate the mechanism of homocysteine induced decrease in adenosine concentration, it has to be emphasized that in most of these experiments, supraphysiological concentrations of homocysteine or homocysteine thiolactone were used and that they do not mimic the situation of hyperhomocysteinemia in man.

More recently, Chen *et al* showed in rats *in vivo* that acute hyperhomocysteinemia, induced by the intravenous infusion of L-homocysteine, as well as chronic hyperhomocysteinemia, induced by a six-week methionine-enriched diet, approximately halved plasma and renal interstitial adenosine concentrations (187).
Recently, we studied for the first time the effect of hyperhomocysteinemia on adenosine concentration and adenosine-induced effects in humans *in vitro* and *in vivo* (159). In isolated human erythrocytes, 100 μmol/l L-homocysteine significantly accelerated cellular uptake of adenosine. This was associated with increased intracellular formation of AdoHcy and could be inhibited by blockade of the equilibrative nucleoside transporter by dipyridamole. Subsequently, we showed that in patients with severe hyperhomocysteinemia due to homozygous deficiency of CBS, forearm vasodilation induced by the infusion of adenosine into the brachial artery was significantly impaired compared with a matched control group. Most probably, this is due to enhanced cellular adenosine uptake as it could be completely restored by coinfusion of the nucleoside uptake inhibitor dipyridamole, which prevents transport of adenosine into the cells and subsequent formation of AdoHcy (159). Based on these observations, we concluded that in these patients, cellular uptake of adenosine is accelerated, thus limiting adenosine receptor stimulation. In sharp contrast to the study by Chen *et al*, (187) baseline plasma and muscle interstitial endogenous adenosine, as measured with microdialysis probes, was not reduced in these patients. This is not surprising, however, when considering the previously mentioned observations that the effect of homocysteine on adenosine and AdoHcy is limited to those situations of increased extracellular concentrations of adenosine, such as in inflammation, hypoxia or ischemia.

In conclusion, several animal studies with experimental hyperhomocysteinemia as well as a study in patients with classical homocystinuria have provided evidence that in these conditions, cellular adenosine uptake is accelerated due to increased intracellular synthesis of AdoHcy. This effect is most pronounced in situations of high extracellular adenosine, such as in inflammation, hypoxia or ischemia, thus limiting adenosine receptor stimulation, aimed at protecting the affected tissue, when most needed.
It remains to be established whether this modulation of adenosine kinetics is also important in patients with mild hyperhomocysteinemia due to thermolabile MTHFR-mutations or vitamin deficiencies, because fluxes of homocysteine are probably different from the situation of CBS deficiency or experimental hyperhomocysteinemia.
These findings on the role of adenosine in hyperhomocysteinemia provide novel theoretical targets for pharmacological intervention in patients with hyperhomocysteinemia, such as nucleoside uptake inhibition. However, more studies on modulation of adenosine by homocysteine need to be conducted in patients with hyperhomocysteinemia *in vivo* before the effects of these pharmacological interventions can be studied in clinical trials.

ACKNOWLEDGEMENTS
Niels P Riksen is a recipient of a fellowship of the Netherlands Organization for Scientific Research (ZonMw). Gerard A Rongen is fellow of the Royal Netherlands Academy of Arts and Sciences (KNAW).

CHAPTER 3.4

ENDOGENOUS ADENOSINE IS NOT INVOLVED IN THE VASODILATOR RESPONSE TO ACUTE ELEVATION OF PLASMA FREE FATTY ACIDS IN HUMANS *IN VIVO*

Submitted

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ABSTRACT

Plasme free fatty acid (FFA) concentrations are elevated in patients with obesity. We aimed to provide an integral hemodynamic profile of elevated plasma FFA by the simultaneous assessment of blood pressure, pulse wave velocity, forearm blood flow (FBF), and sympathetic nervous system activity during acute elevation of FFA. Secondly, we hypothesized that FFA-induced vasodilation is mediated by adenosine receptor stimulation. In a randomized crossover trial in healthy subjects, Intralipid was infused for 2 hours to elevate plasma FFA. Glycerol was administered as Control infusion. We assessed blood pressure, pulse wave velocity, FBF (venous occlusion plethysmography), and sympathetic nervous system activity by measurements of (nor)epinephrine. During the last 15 minutes of Intralipid/Control infusion, the adenosine receptor antagonist caffeine (90 μg/min/dl) was administered into the brachial artery of the nondominant arm. Compared with Control infusion, Intralipid increased pulse wave velocity, systolic blood pressure and pulse pressure, as well as FBF (FBF increased from 1.8±0.2 to 2.7±0.6 and from 2.3±0.2 to 2.7±0.6 ml/min/dl for Intralipid versus Control infusion; $P<0.05$, n=9). Although in a positive control study caffeine attenuated adenosine-induced forearm vasodilation ($P<0.01$, n=6), caffeine had no effect on Intralipid-induced vasodilation ($P=0.5$). In conclusion, elevation of plasma FFA levels increases pulse wave velocity, systolic blood pressure, and pulse pressure. Also, FBF is increased, either by baroreflex-mediated inhibition of the sympathetic nervous system, or by a direct vasodilating effect of FFA. As the adenosine receptor antagonist caffeine could not antagonize the vasodilator response, it is not mediated by adenosine receptor stimulation.

Key words: adenosine, free fatty acids, metabolic syndrome, blood flow, autonomic nervous system.

INTRODUCTION

The prevalence of the metabolic syndrome has strikingly increased over the past two decades, mainly driven by the global epidemic of obesity (231). The metabolic syndrome is characterized by (central) obesity, insulin resistance, glucose intolerance, dyslipidemia, and hypertension. Less well-known but consistently reported features of the metabolic syndrome include a hyperdynamic circulation and sympathetic activation (232-235), and previous studies have shown prospectively that a hyperdynamic circulation predicts future development of type 2 diabetes mellitus (232).

In patients with obesity and the metabolic syndrome, the plasma concentration of free fatty acids (FFA) is increased and evidence is accumulating that FFA play a central role in the clustering of the various characteristics of the metabolic syndrome (121, 236). In the last decade, several studies therefore explored the effect of experimental elevations of plasma FFA on hemodynamic parameters. Systemic administration of a triglyceride emulsion and heparin (to activate the lipoprotein lipase) is a well-validated and generally accepted model to study the effects of an acute elevation of plasma FFA (237-239). Most studies agree that acute experimental elevation of FFA increases skeletal muscle blood flow (237, 240-244). Most (237, 242, 245-247), but not all (240, 244), studies have shown an increase in mean arterial blood pressure. Also the reported effects of FFA on the activity of the sympathetic nervous system are controversial: some studies have demonstrated in rats and humans that acute elevation of FFA can increase sympathetic activity (247, 248), but other studies have reported the opposite (249, 250).

Recently, we presented a novel hypothesis which explains the occurrence of a hyperdynamic circulation and sympathetic activation in individuals with the metabolic syndrome (121). It was postulated that in patients with the metabolic syndrome, elevated cytosolic concentrations of co-enzyme A derivatives of long-chain fatty acids (LCFA-CoA), the intracellular equivalent of FFA, inhibit the mitochondrial adenine nucleotide translocator. Consequently, intra- and extracellular concentrations of the endogenous nucleoside adenosine increase. Adenosine receptor stimulation induces various effects, including vasodilation in most vascular beds, renal sodium retention and activation of the sympathetic nervous system (5, 251). As such, elevated systemic concentrations of adenosine could induce the phenotype of a hyperdynamic circulation and sympathetic activation.

The aim of the present study is twofold: to provide an integral hemodynamic profile of elevated plasma FFA and, more specifically, to study whether adenosine receptor stimulation is involved in the vasodilator response to FFA. Therefore, we performed a randomized controlled cross-over study in healthy volunteers in whom systemic administration of a triglyceride emulsion (Intralipid) was used to elevate plasma FFA, and we simultaneously measured forearm blood flow (FBF), intra-arterial blood pressure, pulse wave velocity, and estimated the activity of the sympathetic nervous system by plasma (nor)epinephrine measurements. In addition, the adenosine receptor antagonist caffeine was administered into the brachial artery of the non-dominant arm to study whether the FFA-induced increase in FBF is due to direct adenosine receptor stimulation in the forearm vascular bed.

METHODS

Subjects

The study conformed with the principles outlined in the Declaration of Helsinki and was approved by the Institutional Review Board of the Radboud University Nijmegen Medical Center. Fifteen young healthy volunteers signed written informed consent before participation. All participants underwent a physical examination and electrocardiography to exclude hypertension and cardiovascular or pulmonary disease. As caffeine is an effective adenosine receptor antagonist (115), all volunteers were asked to abstain from caffeine-containing beverages for at least 24 hours before the study.

Instrumentation

All experiments were performed in the morning after an overnight fast in a temperature-controlled room (23 °C) with the subjects in supine position. After local anesthesia (xylocaine 2%) the brachial artery of the non-dominant arm was cannulated with a 20-gauge catheter (Angiocath, Becton Dickinson, Sandy, UT, USA) for intra-arterial administration of caffeine (automatic syringe infusion pump, type STC-521, Terumo Corporation, Tokyo, Japan) and blood pressure recording (Hewlett Packard GmbH, Böblingen, Germany). FBF was recorded simultaneously on both arms by venous occlusion plethysmography using mercury-in-silastic strain gauges (Hokanson EC4, Hokanson, Inc., Washington, USA) as previously described (141). Before each recording a wrist cuff was inflated to 200 mmHg to exclude the hand circulation.
In the main study two catheters were inserted into large antecubital veins of both arms. Intralipid or glycerol was administered intravenously into the dominant arm (vide infra). Venous blood was drawn from the non-dominant arm for the determination of FFA, glycerol, triglycerides, and glucose. Arterial and venous (nor)epinephrine levels were assessed in blood simultaneously drawn from the brachial artery and antecubital vein from the non-dominant arm. Plasma insulin concentration was determined in arterial blood.

Validation study

Six healthy volunteers participated in the validation study. Thirty minutes after cannulation of the brachial artery of the nondominant arm, FBF was measured for 5 minutes during saline infusion. Then, adenosine was administered into the brachial artery (5 μg/min per dl of forearm tissue) for 10 minutes and FBF was measured. After 20 minutes wash-out, adenosine infusion was repeated, 5 minutes later followed by co-infusion of caffeine (90 μg/min/dl).

Main study

For this study 9 subjects were studied twice according to a randomized crossover design. All subjects were asked to avoid excessive exercise for 24 hours before the tests and to have a similar dinner the evening before both experiments. The second experiment was conducted one month after the first. In one experiment (Intralipid experiment), subjects received an intravenous dose of a triglyceride emulsion (Intralipid® 20%, 90 ml/h) with co-administration of heparin (bolus 500 IU, followed by continuous infusion of 0.2 IU/kg/min intravenously) to activate lipoprotein lipase (237). In the other experiment (Control experiment), glycerol (50% solution; 3 gram/h) and heparin

were administered to control for the elevated glycerol and heparin concentration during the Intralipid experiment. This design has previously been described by Krebs *et al* (252).
In both experiments, FBF was measured for 5 minutes during saline infusion, thirty minutes after cannulation. Thereafter, Intralipid or Control infusion was started. In both experiments, FBF was recorded for 5 minutes and blood was drawn for the determination of FFA, triglycerides and glycerol 60 and 120 minutes after initiation of infusion. Concentrations of insulin and (nor)epinephrine were determined on t=0 and t=120 minutes. Finally, caffeine was administered for 15 minutes into the brachial artery of the nondominant (90 μg/min/dl) during continuous Intralipid or Control infusion and with uninterrupted measurement of FBF.

During the 10 minutes immediately before the start of administration of Intralipid/Control and during the last 10 minutes of infusion, heart rate was recorded by surface electrocardiography, and blood pressure was recorded in the brachial artery. For each heartbeat, we calculated the time difference between the electrocardiographic R wave and arrival of the pressure wave at the brachial artery and averaged this value over these 10 minute periods (MIDAC test-organizer, Radboud University Nijmegen Medical Center). As the travel distance is the same for each subject during the two experiments, this time difference can be considered as a measure for pulse wave velocity.

Analytical procedures
Blood samples for the determination of glycerol, FFA and triglycerides were collected in pre-cooled EDTA-containing vacutainers and centrifuged at 1000 *g* for 10 min at 4°C. Aliquots of plasma were immediately stored at –80°C. FFA (NEFA-C; Wako Chemicals, Neuss, Germany), free glycerol (148270; Roche Diagnostics, Indianapolis, IN), and triglyceride (GPO-Trinder 337B; Sigma Diagnostics, St Louis, MO) concentrations were analyzed with a COBAS semiautomatic analyzer (Roche). Due to technical problems, baseline plasma glucose levels were only determined in our laboratory in the first five experiments.
For the determination of plasma insulin and (nor)epinephrine, blood was collected in pre-cooled lithium-heparin-containing vacutainers. Plasma concentrations of (nor)epinephrine were determined by HPLC with fluorimetric detection (253). Insulin was measured with an insulin-specific double antibody radioimmunoassay (interassay coefficient of variation, 6.2%). Plasma caffeine concentrations were determined by reversed-phase HPLC with UV detection set at 273 nm. Forearm spillover of norepinephrine was estimated as previously described by Rongen *et al* (254). To correct for changes in FBF, we also calculated appearance rate of norepinephrine (254). For these calculations, forearm fractional extraction of epinephrine was used as a substitute for norepinephrine extraction.

Statistical analysis
All data are expressed as mean ± SD, unless otherwise specified. Baseline parameters of subjects in the validation study and main study were compared with an unpaired Student's t-test and baseline values in both experiments of the main study were compared with a paired Student's t-test. Plasma concentrations of free fatty acids, glycerol, triglycerides and insulin as well as hemodynamic parameters in both groups were analyzed by repeated measures ANOVA. Forearm vascular

resistance was calculated by the ratio of mean arterial pressure and FBF. SPSS for Windows (release 12.0.1) was used for statistical analyses.

RESULTS

Baseline characteristics

There were no significant differences between subjects participating in the validation study and the main study. Average age was 21.2 ± 2.3 years. Baseline plasma caffeine concentration was 0.29 ± 0.40 mg/l in the validation study and 0.21 ± 0.1 versus 0.15 ± 0.1 mg/l in the Intralipid and Control study, respectively.

Validation study

Infusion of adenosine into the brachial artery of the non-dominant arm for 5 minutes increased FBF from 1.9 ± 1.1 to 4.0 ± 1.8 ml/min per dl of forearm tissue during the first period of adenosine administration and to 4.1 ± 1.8 ml/min/dl during the second period (figure 1a). In the first period, adenosine infusion was continued resulting in a further increase in FBF up to 6.3 ± 1.0 ml/min/dl. In the second period, caffeine was co-administered after 5 minutes, resulting in a decrease of FBF to 2.9 ± 0.5 ml/min/dl. There was a significant difference between the change in FBF during the first 5 minutes of caffeine administration and the change in FBF observed during continuous infusion of adenosine alone (figure 1b; P<0.01). There were no significant differences between both infusion periods regarding blood pressure, heart rate and FBF in the control arm (data not shown). These results show that caffeine in the dose administered is effective in reducing adenosine-induced vasodilation.

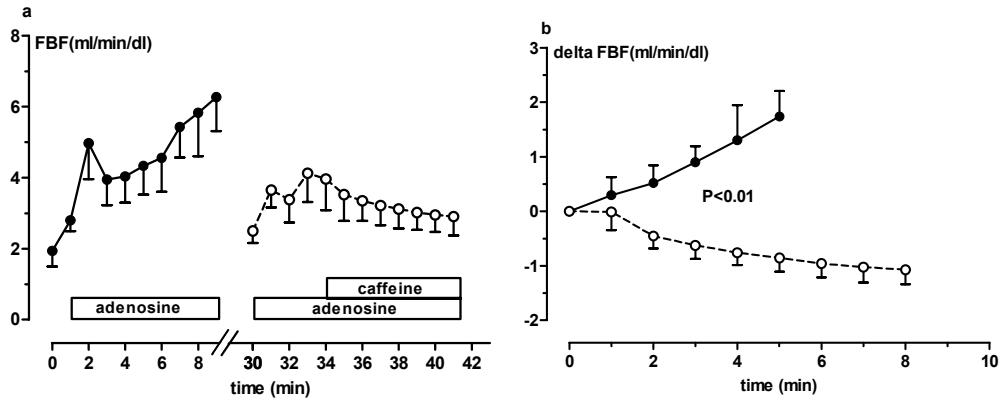

Figure 1: FBF in the experimental arm in the validation study during administration of adenosine (filled circles) and adenosine/caffeine (open circles) in the brachial artery (a, n=6, mean ± SE). Figure 1b depicts the change in FBF after the addition of caffeine (open circles) or continued administration of adenosine only (filled circles). P<0.01 for difference between both interventions, ANOVA for repeated measures.

Main study

Table 1 summarizes the hemodynamic values and plasma concentrations at baseline, and after 1 and 2 hours of Intralipid/Control infusion.

Table 1: Hemodynamic and laboratory values in the main study (mean ± SD; I = Intralipid; C = Control; *P<0.05 for difference between I and C at baseline. #P=0.05 and †P<0.05 for difference between I and C (repeated measures ANOVA).

Variable		Baseline	1 hour	2 hours
FBF (ml/min/dl)	I	1.8 ± 0.7 *	2.1 ± 1.0	2.7 ± 1.8 †
	C	2.3 ± 0.7 *	2.2 ± 0.8	2.7 ± 1.8 †
MAP (mmHg)	I	80 ± 5	81 ± 7	84 ± 7
	C	82 ± 9	83 ± 9	84 ± 9
SBP (mmHg)	I	115 ± 8	119 ± 8	122 ± 8 #
	C	116 ± 10	118 ± 10	120 ± 9 #
DBP (mmHg)	I	62 ± 4	62 ± 7	64 ± 7
	C	63 ± 9	63 ± 8	65 ± 8
Pulse Pressure	I	53 ± 6	58 ± 6	59 ± 6 †
(mmHg)	C	53 ± 5	55 ± 6	55 ± 9 †
Heart rate (bpm)	I	59 ± 6	61 ± 6	65 ± 7
	C	59 ± 6	60 ± 5	63 ± 5
Pulse wave time to	I	174 ± 18		156 ± 17 †
brachial artery (ms)	C	188 ± 55		184 ± 53 †
FFA (µmol/l)	I	334 ± 108	965 ± 198	1269 ± 234
	C	338 ± 114	393 ± 140	432 ± 175
Triglycerides	I	746 ± 216	2824 ± 768	3947 ± 1674
(µmol/l)	C	788 ± 445	842 ± 390	912 ± 412
Glycerol (µmol/l)	I	68 ± 26	333 ± 100	470 ± 140
	C	63 ± 22	301 ± 69	307 ± 128
Glucose (mmol/l)	I	5.0 ± 0.1		4.5 ± 0.5
	C	4.7 ± 0.2		4.5 ± 0.1
Insulin (pmol/l)	I	42.0 ± 16.8		49.2 ± 13.2 †
	C	40.8 ± 12.0		37.8 ± 10.8 †

Plasma concentrations of FFA, triglycerides and glycerol increased significantly more during administration of Intralipid than during Control infusion. During Intralipid infusion a small yet significant increase in plasma insulin concentration was observed (table 1).

Both Intralipid and Control infusion resulted in a significant increase in systolic blood pressure, pulse pressure and heart rate (P<0.01 for all three parameters), whereas diastolic blood pressure and mean arterial pressure were not affected. However, increases in systolic blood pressure and pulse pressure were significantly more pronounced during Intralipid infusion (table 1).

Baseline FBF was significantly higher before Control infusion than before Intralipid infusion. Therefore, we calculated the change in FBF from baseline to express the effects of each intervention. Administration of Intralipid resulted in a significant increase in FBF compared with Control infusion (Figure 2; P<0.05). Also, FVR (MAP/FBF) was significantly decreased during Intralipid infusion compared with Control infusion (from 52.3 ± 15.2 to 40.3 ± 15.9 and from 39.2 ± 10.7 to 43.2 ± 21.2 for Intralipid and Control, respectively, P<0.05).

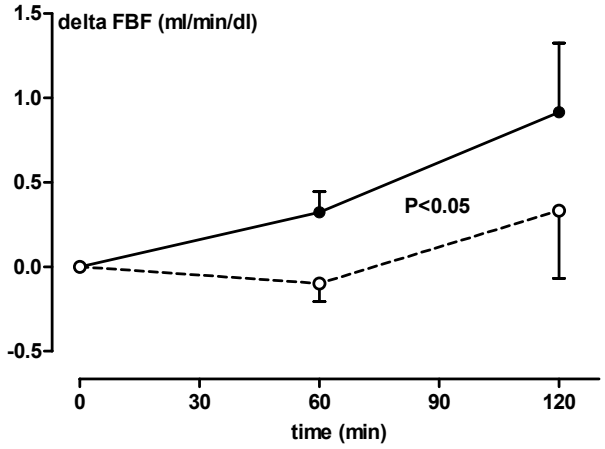

Figure 2: FBF in the experimental arm, expressed as change from baseline, during Intralipid (filled circles) and Control infusion (open circles). P<0.05 for difference between both interventions, ANOVA for repeated measures (n=9, mean ± SE).

The time difference between the electrocardiographic R wave and arrival of the pressure wave at the brachial artery decreased during Intralipid administration compared with Control infusion (table 1), indicating that Intralipid administration significantly increased pulse wave velocity.

Administration of Intralipid had neither an effect on arterial norepinephrine concentration nor on calculated forearm norepinephrine spillover compared with Control infusion. If anything, forearm appearance rate of norepinephrine tended to increase more during Control infusion (table 2).

Table 2: Plasma norepinephrine kinetics (mean ± SD); * P = 0.07 for comparison Intralipid versus Control

Variable	Intralipid T = 0	Intralipid T = 120	Control T = 0	Control T = 120
Arterial norepinephrine (nmol/l)	0.63 ± 0.19	0.71 ± 0.32	0.57 ± 0.22	0.70 ± 0.30
Spillover (pmol/min/dl forearm tissue)	0.66 ± 0.47	0.85 ± 0.64	0.84 ± 0.05	1.06 ± 0.35
Appearance rate (pmol/min/dl forearm tissue)	2.10 ± 1.77	2.82 ± 2.46	2.30 ± 2.11	3.30 ± 1.77*

During continuous systemic administration of Intralipid, infusion of caffeine into the brachial artery of the non-dominant arm did not have any effect on FBF (figure 3; P=0.5 compared with the control arm). Also during continuous Control infusion, caffeine did not affect FBF (data not shown).

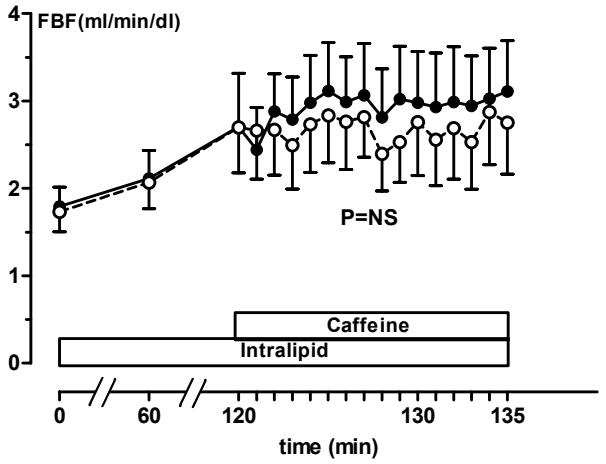

Figure 3: FBF in the experimental (filled circles) and control (open circles) arm during administration of caffeine into the brachial artery for 15 minutes during continuous systemic Intralipid administration. P=0.5 for comparison between both arms, ANOVA for repeated measures (n=9, mean ± SE).

DISCUSSION

The main finding of this study is that acute elevation of FFA induces a hemodynamic profile consisting of increased pulse wave velocity, systolic blood pressure and pulse pressure, and peripheral vasodilation. The observed FFA-induced forearm vasodilation is not mediated by adenosine receptor stimulation.

Previous studies have already shown that a hyperdynamic circulation is associated with several characteristics of the metabolic syndrome, although these findings have not received much attention. In adults a hyperdynamic circulation is associated with increased body mass index, triglyceride levels, glucose and insulin levels (232). Also in children a hyperdynamic circulation is associated with increased triglyceride and insulin levels and with several indices of obesity (234). Interestingly, the presence of a hyperdynamic circulation predicts future development of type 2 diabetes mellitus (232). In addition, insulin resistance and obesity are associated with increased arterial stiffness (255, 256), which is an important cardiovascular risk marker (257).

We hypothesized that this hyperdynamic circulation is caused by increased concentrations of plasma FFA (121). In obese subjects the release of FFA into the circulation is increased as compared to lean subjects (258), and there is accumulating evidence that an abnormal accumulation of triglycerides in muscle and other non-adipose tissues plays a pivotal role in the development of insulin resistance and possibly also in the deterioration of beta-cells in type 2 diabetes (259). Indeed,

we demonstrated that acute experimental elevation of plasma FFA increases systolic blood pressure and pulse pressure, and induces peripheral vasodilation, but we did not observe an increased activity of the sympathetic nervous system. More specifically, we hypothesized that FFA would induce vasodilation by increased adenosine receptor stimulation (121). In animal studies, LCFA-CoA have been shown to result in a concentration-dependent inhibition of the mitochondrial adenine nucleotide translocator in a physiological concentration range (260). The adenine nucleotide translocator transports ADP from the cytosol into the mitochondrial matrix in exchange for ATP. As such, inhibition of this transporter results in increased cytosolic ADP concentrations. Consequently, according to the cytosolic adenylate kinase equilibrium, ATP and AMP concentrations increase and AMP is converted into adenosine. Indeed, it was shown *in vitro* that palmitoyl-CoA inhibits the adenine nucleotide translocator and increases extramitochondrial AMP concentrations (261). An increased intracellular adenosine concentration diminishes the transmembranous adenosine concentration gradient, thus limiting uptake of extracellular adenosine (50). By stimulation of specific membrane-bound adenosine receptors, adenosine induces various cardiovascular and neurohumoral effects (5), such as vasodilation, and activation of the sympathetic nervous system. In addition, some studies suggest that increased systemic adenosine concentrations result in renal sodium and water retention (251). Together, these findings suggest a role for adenosine in causing the hemodynamic changes observed in the metabolic syndrome and in response to administration of FFA.

It has been demonstrated before that acute elevation of FFA is associated with increased forearm and leg blood flow (237, 240-244). In the present study, we confirmed this vasodilator effect. Subsequently, we administered the adenosine receptor antagonist caffeine into the brachial artery of the non-dominant arm in order to explore the role of local adenosine receptor stimulation in this FFA-induced vasodilation. Administration of caffeine did not affect FBF in this setting, whereas in the same dose it effectively blocked adenosine-induced vasodilation. As such, our findings do not support a role for adenosine in the vasodilator action of FFA.

In the present study, experimental elevation of FFA was not associated with activation of the sympathetic nervous system. If anything, a reduction of forearm appearance rate of norepinephrine was observed. Previous studies on this subject in healthy subjects yielded inconsistent results (247, 250). Also in rat models, administration of lipid emulsions has been demonstrated to either increase (248) or decrease (249) sympathetic activity. Because of these inconsistent results, it remains to be determined whether FFA are responsible for the reported association between the metabolic syndrome and sympathetic activation.

Interestingly, we also demonstrated that administration of Intralipid decreased the time difference between the electrocardiographic R wave and arrival of the pressure wave at the brachial artery. As the travel distance is the same for each subject during both experiments, this observation implies that elevation of FFA increases pulse wave velocity in large arteries. This is in accordance with the results of two previous studies in which compliance after administration of a triglyceride emulsion was estimated by pulse wave analysis (246, 262).

A plausible explanation for the hemodynamic effects of FFA in our study is that FFA reduced arterial compliance, which increased systolic blood pressure and pulse pressure by impairing windkessel function and by summation of the propagated and reflected pressure waves. Subsequently, baroreflex-mediated inhibition of the sympathetic nervous system results in peripheral vasodilation. A concomitant reduction in heart rate would have been expected in this situation, but it has been demonstrated in rats (263) and humans (262) that acute experimental elevation of plasma FFA reduces baroreflex sensitivity, which is considered mainly to reflect vagal activity. An alternative explanation would be that FFA also have a direct vasodilating effect on resistance arteries, not mediated by adenosine receptor stimulation, in addition to the reduction in arterial compliance.

A number of potential limitations of our study should be mentioned. First, the study was not blinded. Because of the different color of the Intralipid emulsion and glycerol solution blinding of this study would have been extremely complicated. However, the test sequence was randomized, and the hemodynamic and laboratory analyses were performed blinded. Second, plasma insulin levels were slightly but significantly increased by administration of Intralipid compared with Control. This is in accordance with the well-known effect of FFA on insulin-mediated glucose uptake (252). It is unlikely that this increased insulin concentration contributed to the increase in FBF because in previous studies with comparable study protocols, insulin either slightly increased (240) or did not increase at all (244), whereas blood flow increased in all studies. Moreover, insulin-mediated vasodilation was observed in previous studies only after much higher elevations of plasma insulin of approximately ten-times baseline levels (264). Thirdly, baseline FBF was significantly higher before Control infusion than before Intralipid infusion. However, it is unlikely that this affected the vasomotor response to Intralipid/Control infusion, as there was no correlation between baseline FBF and the change in FBF in response to Intralipid or Control infusion (data not shown). Finally, it has to be realized that we studied acute and not chronic elevation of plasma FFA and that the plasma FFA concentration after Intralipid infusion was higher than the FFA concentration in patients with the metabolic syndrome or diabetes mellitus (265). Thus, future studies are warranted to confirm that the hemodynamic effects as observed in the present study also apply to chronic and less extreme elevations in plasma FFA concentration.

In conclusion, acute elevation of plasma FFA levels in healthy young subjects increases pulse wave velocity, systolic blood pressure and pulse pressure, decreases forearm vascular resistance, but does not significantly increase the activity of the sympathetic nervous system. The reduced vascular resistance could be due to baroreflex-mediated inhibition of sympathetic nerve activity. Alternatively, FFA could have direct vasodilator effects, which, however, are not mediated by local adenosine receptor stimulation.

ACKNOWLEDGEMENTS

The technical support by Petra van den Broek (Pharmacology-Toxicology, Radboud University Nijmegen Medical Center), and Dr. Luc J van Loon (Human Biology and Movement Sciences, Nutrition Research Institute Maastricht, Maastricht University) is gratefully acknowledged. The study was supported by a grant of the Dutch Diabetes Foundation (grant 1999.007). NP Riksen is a recipient of an AGIKO-fellowship of the Netherlands Organization for Scientific Research (ZonMw). CJ Tack is a recipient of a clinical fellowship of the Dutch Diabetes Foundation. The contribution of GA Rongen has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences (KNAW).

CHAPTER 4

PHARMACOLOGICAL MODULATION OF THE CARDIOVASCULAR EFFECTS OF ADENOSINE

CHAPTER 4.1

ISCHEMIC PRECONDITIONING: FROM MOLECULAR CHARACTERIZATION TO CLINICAL APPLICATION

PART I

Neth J Med 2004;62:353-63

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ABSTRACT

Ischemic preconditioning is defined as an increased tolerance to ischemia and reperfusion induced by a previous sublethal period of ischemia. Since this mechanism is the most powerful infarct-size limiting mechanism, other than timely reperfusion, an overwhelming amount of studies have addressed the mechanisms by which this form of protection occurs. During the short preconditioning period of ischemia, several trigger substances are released (adenosine, bradykinin, norepinephrine, opioids). By activation of membrane-bound receptors, these substances activate a complex intracellular signalling cascade, which converges on mitochondrial end-effectors, including the ATP-sensitive potassium-channel and the mitochondrial permeability transition pore. Activation of this pathway protects cardiomyocytes against both necrosis and apoptosis during a subsequent more prolonged ischemic episode. The protection afforded by preconditioning lasts only 2-3 hours, but reappears 24 hours after the preconditioning stimulus. This "delayed preconditioning" requires synthesis of new proteins, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and heat shock proteins. Additionally, preconditioning is not confined to one organ, but can also limit infarct-size in remote, non-preconditioned organs ("remote preconditioning").

Knowledge of these mechanisms that mediate ischemic preconditioning is essential to understand which drugs are able to mimic preconditioning or interfere with preconditioning in patients at risk for myocardial ischemia. This review aims to summarize current knowledge regarding the different forms and mechanisms of ischemic preconditioning.

INTRODUCTION

Despite major advances in prevention and treatment, ischemic heart disease and in particular acute myocardial infarction with its late sequelae, remain the leading cause of morbidity and mortality in the Western world and is rapidly gaining its leading position in the developing world (266). Moreover, due to improved survival from acute myocardial infarction, more and more patients suffer from chronic heart failure, which is an important late complication of infarction. In this regard, continued improvement of strategies aimed at primary and secondary prevention of myocardial infarction is essential. To define suitable targets for intervention, three factors can be identified that ultimately determine the development and outcome of coronary occlusion (267, 268). The occurrence of coronary artery occlusion is determined by "vulnerable plaques" (prone to thrombotic complications) and "vulnerable blood" (prone to thrombosis). Once coronary occlusion has occurred, the clinical outcome is dependent on the "vulnerability" of the myocardium. Complementary to primary prevention, limitation of infarct size, once occlusion has occurred, is an interesting target, which could ultimately attenuate the development of subsequent heart failure.

Until 1986 it was unknown whether therapeutic infarct size limitation was possible at all. In that year, the landmark study by Murry *et al* was published, in which they described that brief periods of ischemia (preconditioning ischemia) in a dog model rendered the myocardium resistant to a subsequent more prolonged ischemic period (index ischemia), since than known as "ischemic preconditioning" (122). Four cycles of 5 minutes coronary occlusion prior to 40 minutes occlusion reduced infarct-size induced by these 40 minutes of occlusion by 75% (figure 1).

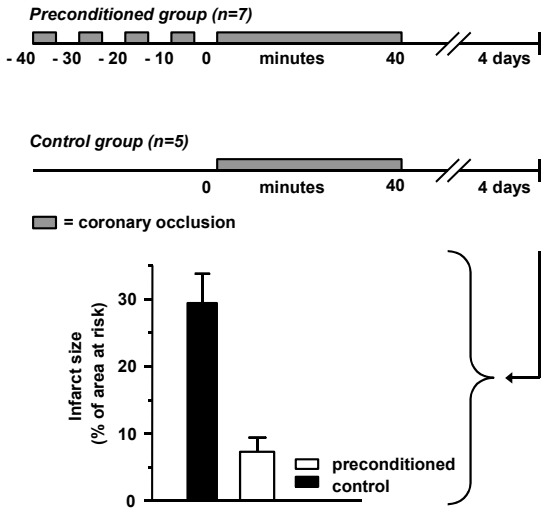

Figure 1: Protocol and results of the original study by Murry *et al*, who showed that in the dog heart, 4 cycles of 5-minute coronary occlusion reduced infarct-size induced by a subsequent 40-minute coronary occlusion and histologically assessed after 4 days of reperfusion, by 75%.

However, the infarct sparing effect was lost when 3 hours of occlusion was applied, emphasizing that timely reperfusion remains indispensable for preconditioning to limit myocardial damage. Since than, an overwhelming amount of studies investigated the underlying mechanism, with the ultimate aim of exploiting this powerful protective mechanism in clinical practice. It was found that ischemic preconditioning offers two windows of protection in time, called "early" or "classical" preconditioning, providing protection immediately after the preconditioning stimulus, and "late" or "delayed" preconditioning (269). Also, it was found that preconditioning ischemia is able to protect remote cells and organs, which have not been preconditioned by itself ("remote preconditioning") (270, 271). It is essential to realize that most of these studies have been conducted in animal models and that important inter-species differences might exist concerning the mechanism of protection, although the effect of preconditioning could be reproduced in all species studied so far (272). In addition, in time various *in vitro* and *in vivo* human models have been developed, often using surrogate end-points to study the effect of preconditioning (273).
This review represents the first part of two that deal with ischemic preconditioning. In this first part, we focus on the mechanisms, which are responsible for ischemic preconditioning. Knowledge of these signalling cascades is essential to understand how various drugs could mimic ischemic preconditioning or interfere with ischemic preconditioning. Indeed, many drugs that are currently used in clinical practice have the potential of interfering with ischemic preconditioning, which is especially relevant in patients who are at risk for ischemia. In the second part we will focus on this pharmacological modulation of ischemic preconditioning and we will describe the potential therapeutic applications of preconditioning in the near future (274).

EARLY ISCHEMIC PRECONDITIONING

In the original paper by Murry *et al*, it was described that ischemic preconditioning reduced infarct size, expressed as percentage of the area at risk, by approximately 75% (122). Ever since, this remained the primary end-point to describe the effect of ischemic preconditioning. Moreover, using this end-point, classical preconditioning limited infarct size in every species tested so far. That this infarct-size limitation indeed would be able to attenuate the progression to heart failure after myocardial infarction is suggested by the study by Cohen *et al,* who showed that in rabbits early ischemic preconditioning not only reduced infarct size, but also improved systolic myocardial function, measured three weeks after the index ischemic insult (275). For studying ischemic preconditioning in humans, especially *in vivo*, several surrogate end-points have been developed, such as ECG changes and coronary lactate, which will be discussed in more detail in the second part of the review.
Besides infarct-size limitation, ischemic preconditioning has also been shown to attenuate other forms of ischemic injury, such as stunning and ventricular arrhythmias, although the evidence is less convincing than for infarct-size limitation (272, 276). In the present review, we will focus primarily on necrosis and apoptosis of cardiomyocytes as primary end-point of ischemia and reperfusion injury.

The duration of the preconditioning ischemia as well as the period of reperfusion before the index ischemia is applied, show fairly rigid time frames in order to give full protection. Concerning the preconditioning ischemic period, protection has been described for periods ranging from one cycle

of 1.25 minutes (277) to five 5-minute ischemia/5-minute reperfusion cycles (278). It is important to realize that the nature of the preconditioning ischemic stimulus (amount and duration of ischemic episodes) influences not only the amount of protection as well as the signalling pathways involved (277, 279). Too many repetitive stimuli might actually abolish preconditioning (280). Concerning the reperfusion period before the index ischemia is applied, the minimum duration lies between 30 seconds and 1 minute (281) and when the reperfusion period is extended beyond 1-2 hours, the infarct limiting effect is no longer evident (282, 283). At this point, it is interesting to mention that in animal models also triggers other than complete ischemia are able to bring myocardium into the preconditioned state. The observation that myocardium can also be preconditioned by a partial coronary occlusion without reperfusion preceding a sustained period of total occlusion, has potential clinical significance considering the nature of thrombus formation in acute myocardial infarction (284). Also, a brief period of acute volume loading resulting in myocardial stretch (285, 286), a brief period of rapid pacing (287) or transient hyperthermia (288) preceding a sustained period of myocardial ischemia have all been shown to limit infarct-size, sharing largely similar signalling pathways as classic ischemic preconditioning.

In recent years, much research has been devoted to elucidate the mechanisms, which are responsible for the preconditioning-induced protection against ischemia-reperfusion injury. When considering the signalling cascade, triggers and mediators, which ultimately converge on end-effectors, can be differentiated. Triggers are released during the short preconditioning ischemia and exert their activity only during this period, whereas end-effectors are solely active during the prolonged index ischemia and actually cause the protection when needed (figure 2).
The first-identified and probably most important trigger of classic preconditioning is the endogenous nucleoside adenosine. Myocardial interstitial adenosine concentration increases rapidly during ischemia (56). In 1991 it was discovered that adenosine A_1 receptor stimulation during the preconditioning ischemia is essential for protection to occur (86) and that intravenous administration of selective adenosine A_1 receptor agonists instead of the preconditioning ischemia offered similar protection (pharmacological preconditioning) (203). Similarly, local intracoronary adenosine administration offered protection similar to ischemic preconditioning in dog hearts (289). Later it was found both *in vitro* and *in vivo* that A_3 receptor stimulation also contributes to ischemic preconditioning (87, 279). Additional evidence for an important role for adenosine as a trigger of early preconditioning is derived from the observation that pharmacological potentiation of the ischemia-induced increase in adenosine concentration during preconditioning, by pre-treatment with the adenosine-uptake inhibitor dipyridamole significantly increases the infarct-size limiting effect of preconditioning (191).

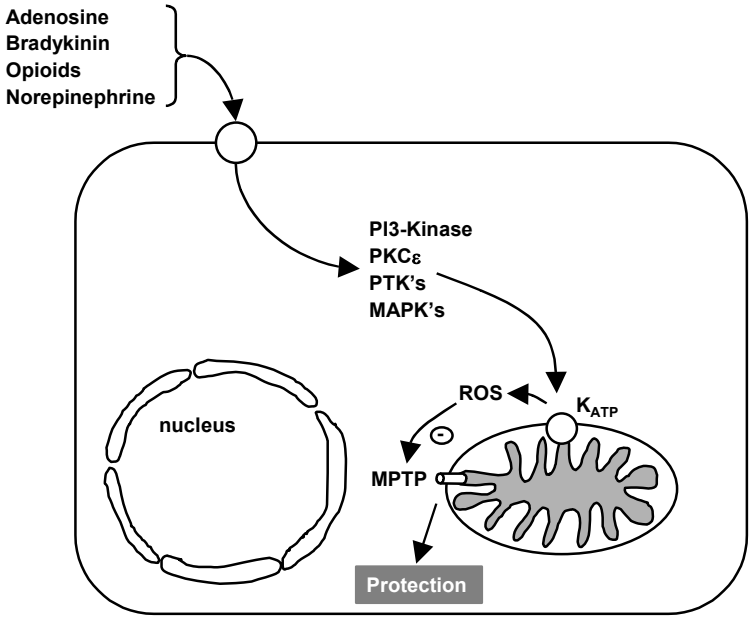

Figure 2: Simplified representation of the mechanism of classical preconditioning. During the preconditioning stimulus, several triggers are released which activate a complex signalling cascade, including phosphatidyl-inositol-3-kinase (PI3-kinase), protein kinase C (PKC), protein tyrosine kinases (PTK) and mitogen-activated-protein kinases (MAPK's). This signalling cascade inhibits opening of the MPTP via mitoK_{ATP}-channel opening and ROS formation.

Considering the protective role of adenosine in ischemia-reperfusion injury, it is important to realize that, in addition to its role as a trigger of ischemic preconditioning, endogenous adenosine also provides direct protection against both ischemia and reperfusion injury, independent of preconditioning, which involves stimulation of adenosine A_{2A} receptors (figure 3)(8). Later it was found that, in addition to adenosine, several other trigger substances, like bradykinin (290), opioids (291), norepinephrine (292) and reactive oxygen species (ROS) (293) are released during preconditioning ischemia and contribute to the infarct sparing effect. Regarding ROS, this seems paradoxical, as ROS are generally assumed to contribute to ischemia-reperfusion injury. Indeed, ROS act as a trigger to protection during the preconditioning stimulus, whereas during the index ischemia and reperfusion, it contributes to injury (294). Also, a transient elevation in calcium during the preconditioning stimulus might contribute to the protection observed (295). Whereas an important role for nitric oxide (NO) has unequivocally been shown in delayed preconditioning, its role in classic preconditioning is more controversial. Although exogenous administration of NO donors prior to ischemia can limit infarct-size, endogenous NO-synthase derived NO is probably not involved in classic preconditioning (296).

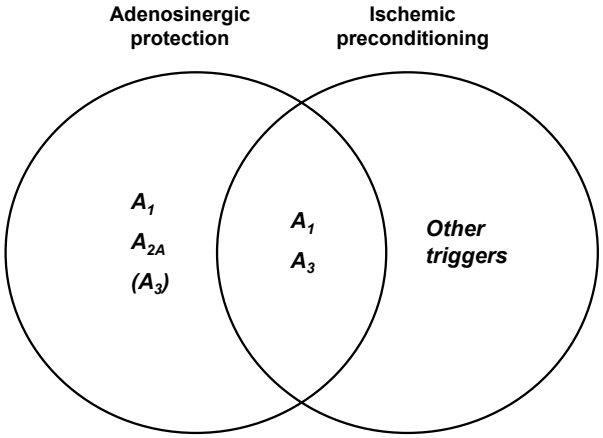

Figure 3: Simplified illustration of the cardioprotection by endogenous adenosine. In addition to the protection afforded by ischemic preconditioning, adenosine also provides direct cardioprotection during ischemia and reperfusion.

It is suggested that because of this redundancy concerning the preconditioning stimulus, blockade of one single receptor type only raise the ischemic threshold required to provide protection, rather than completely block protection (290). Moreover, several studies suggest that the contribution of each of these trigger substances to the induction of preconditioning depends on the nature of the stimulus, which is important to realize when comparing results of different study protocols (279, 297).
As previously mentioned, it is also possible to pharmacologically precondition myocardium. Besides the abovementioned triggers this can also be achieved with norepinephrine (298), endothelin-1 (299), acetylcholine (300) and angiotensin II (301), but these substances are not released in sufficient quantity during ischemia to contribute to endogenous protection.

After this triggering phase, an intracellular cascade of events finally brings the cell into its protected phenotype (figure 2). Several essential components of this cascade have been identified, although the exact sequence is not yet fully elucidated. The activation of the intracellular enzyme protein kinase C (PKC) is essential for ischemic preconditioning (302, 303). Several studies showed that PKC-activation is mediated via activation of phosphatidylinositol-3-kinase (PI3K), which is an important upstream signalling molecule (304, 305). PI3K activates the serine/threonine kinase Akt, which subsequently inactivates the pro-apoptotic kinase glycogen synthase kinase-3 (GSK-3) (306).
Following activation, PKC actually translocates from the cytosol to the particulate fraction where phosphorylation of specific substrates can occur (307). Specific activation and translocation of the isoform PKC-ε seems to be responsible for ischemic preconditioning (308). Interestingly, in some animal models only inhibition of PKC during the index ischemia aborts preconditioning, suggesting that PKC is a mediator and not a trigger (309). Additionally, activation of a tyrosine kinase mediates early preconditioning, either downstream (310) or in parallel with PKC (311). Also,

each subfamily of the mitogen-activated protein kinases (MAPK's), namely the 42/44-kDa extracellular receptor kinase (ERK), the 46/54-kDa c-*jun* kinase (JNK) and the 38-kDa p38 MAPK has been proposed to be involved in the signalling cascade of ischemic preconditioning (reviewed in (312) and (313)).

Another essential component of the mechanism leading to early protection after preconditioning is the ATP-sensitive potassium channel (K_{ATP}-channel). This channel, which opens when intracellular levels of ATP decline, is the known target of sulfonylureas in the pancreas, but is also present in cardiomyocytes and vascular smooth muscle cells. Cardiomyocytes contain K_{ATP}-channels located on both the sarcolemma (sarcK_{ATP}-channels) and the mitochondrial membrane (mitoK_{ATP}-channels). These channels have different pharmacological profiles (294). Both channels are blocked by glibenclamide whereas the mitoK_{ATP}-channel is selectively blocked by 5-hydroxydecanoate (5-HD). Diazoxide opens the mitoK_{ATP}-channel with far greater affinity than the sarcK_{ATP}-channels. Gross and Auchampach firstly described the critical role of K_{ATP}-channel opening in ischemic preconditioning, because early preconditioning was completely inhibited by the administration of glibenclamide either before or immediately after the preconditioning ischemia (314). Initially, sarcK_{ATP}-channels were held responsible for preconditioning, but recent evidence increasingly favours a role for mitoK_{ATP}-channels (extensively reviewed in (294, 315, 316)). Several studies showed that the administration of diazoxide is able to mimic ischemic preconditioning (317, 318) and that 5-HD inhibits preconditioning (319). However, some recent studies still suggest that sarcoK_{ATP}-channels are also involved (320). It appears likely that opening mitoK_{ATP}-channels is not only an end-effector of preconditioning, but also a trigger, as opening is also essential during the preconditioning stimulus (294).

Which end-effectors are involved and how these end-effectors ultimately provide protection is the most elusive part of ischemic preconditioning. Inhibition of the sodium/hydrogen exchanger, prevention of osmotic swelling and prevention of cytoskeleton disruption by heat shock protein HSP27 have all been proposed to act as end-effectors (272, 321). Lately, however, accumulating evidence strongly suggest that the various upstream signalling pathways all converge on mitochondrial proteins aimed at limiting in particular reperfusion injury. In order to adequately understand this complex part of the preconditioning cascade, we will briefly focus on mitochondrial function, with particular emphasis on the role of mitochondria in reperfusion injury. Although reperfusion is essential for cardiomyocytes to survive a period of ischemia, it is well appreciated that reperfusion itself can expedite cell death, which is known as reperfusion injury (322). The mechanism of reperfusion injury differs from ischemic injury, best illustrated by the role of apoptosis in both forms of injury. The vast majority of studies on this topic conclude that apoptosis, in contrast to necrotic cell death, only occurs, or is accelerated, during reperfusion and not during ischemia (323, 324). Reperfusion is characterized by a boost of ROS, which are important mediators of reperfusion injury, as anti-oxidants, applied during reperfusion, limit cellular death (325). Moreover, as apoptosis is an energy-requiring form of cell death, it has been postulated that reperfusion is essential to generate the necessary amount of adenosine-tri-phosphate (ATP) molecules (326). Mitochondria play a prominent role in reperfusion. The most important function of mitochondria is the generation of ATP, by the transfer of electrons on oxygen (327). This transfer

is associated with a transfer of H^+-ions from the inside to the outside of the mitochondrial inner membrane, thus establishing the mitochondrial transmembrane potential. Subsequently, the passive inward flux of H^+-ions forms the driving force for ATP production. Moreover, during electron transfer, 1-5% of ions lose their way and participate in the formation of ROS (327). The mitochondrial permeability transition pore (MPTP) is formed by multiprotein complexes capable of forming large non-selective pores in the otherwise highly impermeable inner mitochondrial membrane (328). There is a large body of evidence that this pore, which remains closed during ischemia, opens during reperfusion (329). This pore is characteristically opened by high mitochondrial [Ca^{2+}], oxidative stress, ATP depletion and mitochondrial depolarization, all pre-eminently present during reperfusion (330). Mitochondrial permeability transition during reperfusion results in uncoupling of the respiratory chain, ultimately resulting in ATP depletion and necrosis on the one hand and in matrix swelling and subsequent rupture of the outer membrane, resulting in release of pro-apoptotic proteins and apoptosis on the other hand (330). That opening of the MPTP indeed contributes to reperfusion injury, is convincingly demonstrated by showing that inhibition of MPTP opening at reperfusion, typically with cyclosporine A (CsA), significantly reduces ischemia-reperfusion injury (reviewed in (330)).
A series of recent studies showed that ischemic and pharmacological preconditioning ultimately provide protection by inhibiting ROS induced opening of the MPTP during reperfusion (331-335). Very recently, an extensive and elegant study by Juhaszova *et al* showed that ischemic preconditioning as well as pharmacological preconditioning by a wide variety of drugs act by inhibiting ROS-induced MPTP opening at reperfusion and this study elucidated great part of the signalling cascade responsible for MPT inhibition (336). They showed that cardioprotection with a memory (e.g. by ischemia, diazoxide, pinacidil, bradykinin) opens mitoK_{ATP}-channels, resulting in a subtle mitochondrial swelling, which increases electron transport and gives rise to a small burst of ROS production, which acts as a messenger to activate PKC, which ultimately converge on phosphorylation of GSK-3β. Phosphorylation of GSK-3β inhibits its function and inhibits MPTP opening during reperfusion. Interestingly, GSK-3β can be inhibited by lithium, which has previously been shown to reduce infarct size (306).

In conclusion, the infarct-limiting effect of ischemic preconditioning seems to be largely mediated by inhibition of reperfusion injury and subsequent apoptosis. There is convincing evidence that in myocardial infarction, both necrosis and apoptosis are involved (reviewed in (337)). Various animal studies showed significant reduction in myocardial infarct-size using inhibitors of apoptosis, such as caspase inhibitors, during reperfusion (338-341). Moreover, caspase or endonuclease inhibition after myocardial infarction attenuates ventricular remodelling and improves contractile function (338, 342). Gottlieb *et al* were the first to show that in an *in vitro* model of rabbit cardiomyocytes, ischemic preconditioning inhibits ischemia-reperfusion-induced apoptosis (343). Later, this was confirmed *in vivo* in a rat model of myocardial ischemic preconditioning (278).

With increasing emphasis on the pivotal role of limitation of reperfusion injury in the infarct-size limitation by ischemic preconditioning, several studies explored whether interventions during reperfusion, rather than before ischemia, could also limit infarct-size. This is of great potential importance, as ischemic insults are seldomly predictable and therefore interventions at the time of

reperfusion are more suited to most clinical scenarios. Indeed, intermittent short repetitive interruptions to reperfusion at the very onset of reperfusion were shown to provide similar protection as ischemic preconditioning in dogs and rats, via activation of the PI3K-Akt pathway (344, 345) (reviewed in (346)).

DELAYED ISCHEMIC PRECONDITIONING.

In 1993, it was first described that the protective effect of ischemic preconditioning, which was previously thought to be a transient phenomenon, reappears 24 hours after the preconditioning ischemic period and results in a delayed protected phenotype (269, 347). Although not as powerful as the early protection provided by preconditioning (infarct size reduction on average 50% (269, 348)), this delayed phase of protection lasts up to 72 hours and, in that respect, might be more therapeutically applicable in clinical practice (348). Moreover, this late phase of preconditioning also provides robust protection against myocardial stunning (349). This delayed phase of protection is also called "late" preconditioning or the "second window of protection" (SWOP).

Although classical and delayed protection largely share common signalling pathways, several essential differences are present (Figure 4).

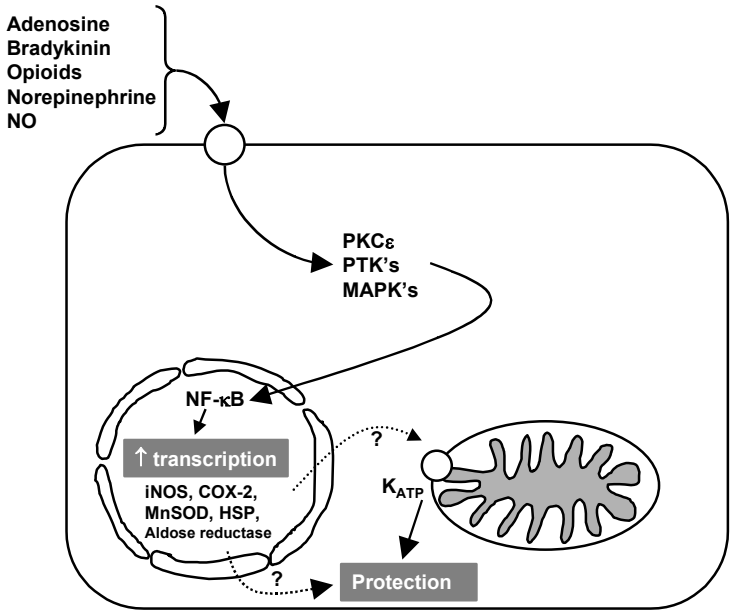

Figure 4: Schematical illustration of the mechanism of delayed preconditioning. In contrast to classical preconditioning, nitric oxide (NO) is an important trigger of delayed protection. Activation of the transcriptional regulator nuclear factor-κB (NF-κB) causes increased transcription of several proteins. Opening of mitochondrial K_{ATP} channels is necessary for the ultimate infarct-size limitation, but how these channels are opened is still a matter of debate.

In this review, we only briefly highlight the differences between classical and delayed preconditioning, the latter being more extensively reviewed elsewhere (272, 350). The distinctive time course of delayed preconditioning and its complete inhibition by protein synthesis inhibitors (351), suggest that synthesis of new proteins is required to obtain the protected phenotype, which is the most striking difference between classical and delayed preconditioning. It is important to realize that the mechanisms mediating protection against infarction and against stunning are not the same, although many pathways are shared, evidenced by the fact that adenosine and K_{ATP}-channels play an obligatory role in protection against infarction (352, 353), but not against stunning (354).

The most important difference between early and late preconditioning regarding the trigger phase is that in delayed preconditioning, in addition to the triggers which are also active in classical preconditioning, endogenous nitric oxide (NO) also provides delayed protection against both stunning and infarction, most likely being derived from endothelial NO synthase (eNOS) (355, 356). Subsequently, these triggers initiate a signalling cascade ultimately resulting in increased transcription of cardioprotective genes. Indispensable for this cascade are PKC (357) and, probably downstream to PKC, tyrosine kinases (358) and most likely also other protein kinases, which activate the important transcriptional regulator nuclear factor-κB (NF-κB) (359). Consequently, increased transcription of protective proteins occurs, several of which have been identified so far. Interestingly, NO synthase is also essential during the index ischemic insult for delayed protection to occur. However, in contrast to the trigger phase, in which eNOS is probably involved, during the index ischemia inducible NOS (iNOS) is upregulated and inhibition of iNOS during this index ischemia completely abrogates protection (360). Similarly, selective inhibition during the index ischemia of cyclooxygenase (COX)-2, which was upregulated 24 hours after the preconditioning stimulus, completely blocked protection against stunning as well as infarction (361). Other proteins, which are upregulated and are important in delayed preconditioning, are superoxide dismutase, which is an important anti-oxidant enzyme (362), and heat shock proteins, although concerning the latter, controversy still exists (272). How these upregulated proteins subsequently provide protection to ischemic injury has not yet been unraveled. However, there is evidence that activation of protein tyrosine kinases is also necessary during the index ischemia for protection to occur, suggesting a role for post-translation modification of the upregulated proteins (363). Finally, it is known that opening of K_{ATP}-channels during the index ischemia is necessary for the infarct-sparing effect of delayed preconditioning, whereas delayed protection to stunning does not seem to require K_{ATP}-channel opening (364). The observation that 5-HD during the preconditioning ischemia inhibits delayed protection, favours a role for the mitoK_{ATP}-channel rather than the sarcolemmal K_{ATP}-channels (365). Although K_{ATP}-channel opening seems to be a final common pathway on which the signalling cascades converge, it is not yet well understood how opening of these channels provides protection.

Similar to early preconditioning, several pharmacological interventions are able to trigger delayed protection, mimicking ischemic preconditioning. In this regard, brief exposure to selective adenosine A_1 and A_3 receptor agonists, exogenous NO donors, ROS-generating substances, bradykinin, δ-opioid agonists and norepinephrine provide delayed protection to infarction (272). This offers possibilities for future exploitation of this delayed mechanism in clinical practice.

REMOTE ISCHEMIC PRECONDITIONING

In 1993, Przyklenk *et al* extended the initial view on ischemic preconditioning tremendously by demonstrating that brief preconditioning occlusions of the circumflex artery could also limit infarct size from subsequent sustained occlusion of the left anterior descending artery in the dog heart (270). This was called "remote intra-cardiac preconditioning". Later, it was shown that remote ischemic preconditioning was not limited to one particular organ system. Transient occlusions of the mesenteric artery limited myocardial infarct-size by a subsequent prolonged coronary occlusion (271, 366), since than known as "inter-organ preconditioning", "remote preconditioning" or "preconditioning at a distance". Since this original finding, remote ischemic preconditioning of the myocardium has been accomplished by transient circulatory occlusion of the short bowel (271, 367), kidney (368) and hind limb (369, 370), but not of the brain (371). Similarly, preconditioning the limb in a pig model limited infarct-size in several remote skeletal muscles after a subsequent prolonged ischemia (372) and transient ischemia of the liver rendered the kidney more resistant to subsequent more severe ischemia in rats (373).

Early remote ischemic preconditioning has been shown in rats (271), rabbits (374) and pigs (375), limiting myocardial infarct-size to a similar extent as classical preconditioning (271, 370, 376). Additionally, a second window of remote protection of the myocardium by applying a short period of preconditioning ischemia to the small intestine has been shown in rats and rabbits (374, 377, 378). The mechanism underlying remote ischemic preconditioning is not yet as well defined as the mechanisms mediating classic preconditioning. Interestingly, in the first study on inter-organ remote preconditioning, Gho *et al* already identified two important clues for understanding the mechanism of protection (271). First, ganglion blockade with hexamethonium prior to the preconditioning stimulus abolished cardioprotection, suggesting neural involvement. Secondly, reperfusion after the preconditioning ischemia was essential, suggesting that at reperfusion substances are released in the mesenteric bed that stimulate afferent neurofibers or directly protect the heart. Although several other studies confirmed involvement of a neurogenic pathway in mesenteric preconditioning of the myocardium (367, 379), preconditioning with a more prolonged mesenteric occlusion was not abolished by hexamethonium (380). Additional evidence that a humoral factor is also involved in remote preconditioning comes from the observation that in rabbits cardioprotection by a preceding short period of coronary occlusion can be transferred to a non-preconditioned heart via coronary effluent transfusion and even transfusion of whole blood (381-383). This transferred protection is not mediated via adenosine or norepinephrine in the effluent and can be abolished by the opioid-antagonist naloxone. Additional studies on mesenteric preconditioning of the myocardium showed that capsaicine-sensitive sensory nerves might be involved (374) and that the protection is abolished by pre-treatment with naloxone and a bradykinin receptor-antagonist (379) before the transient mesenteric occlusion (384). Moreover, signal transduction via PKC is proposed, based on the findings that inhibition of PKC before as well as after the preconditioning stimulus inhibits protection and that brief mesenteric artery occlusion induces a rapid translocation of PKC-ε from the cytosol to membrane fractions in cardiomyocytes (380, 385). In a rabbit model, it was shown that cardioprotection by a brief renal artery occlusion was totally abolished by adenosine antagonism either before the renal occlusion or before the subsequent coronary occlusion, proposing a dual role for adenosine as trigger and mediator of remote preconditioning (370, 376). In line with these observations, Liem *et al* recently

described evidence that in remote preconditioning small intestine ischemia, locally released adenosine triggers afferent nerves which in turn lead to stimulation of cardiac adenosine receptors (367). Finally, very limited evidence suggests that remote preconditioning also occurs in humans *in vivo*, using a surrogate marker of ischemic damage. Kharbanda *et al* showed that three 5-minute cycles of forearm ischemia prevents the reduction in acetylcholine-induced vasodilation after 20 minutes of ischemia of the contralateral arm (375).

ACKNOWLEDGEMENTS

N.P. Riksen is a MD-clinical research trainee financially supported by the Netherlands Organization of Scientific Research (ZonMw). The contribution of G.A. Rongen has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences.

CHAPTER 4.2

ISCHEMIC PRECONDITIONING: FROM MOLECULAR CHARACTERIZATION TO CLINICAL APPLICATION

PART II

Neth J Med 2004;62:409-23

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ABSTRACT

Ischemic preconditioning is originally described in animal hearts as histological infarct-size limitation by a previous brief episode of ischemia. In humans, ischemic preconditioning has been demonstrated in several *in vitro* and *in vivo* models, including coronary artery bypass grafting and percutaneous transluminal coronary angiography, using surrogate markers of ischemia and reperfusion injury. Increasing knowledge of the molecular signalling pathways mediating protection by ischemic preconditioning has provided rational targets for pharmacological intervention. Several widely used drugs are able to mimic ischemic preconditioning (e.g. adenosine, adenosine-uptake inhibitors, ACE-inhibitors, angiotensin-II antagonists, statins, opioids, volatile anesthetics and ethanol), whereas others inhibit ischemic preconditioning-induced protection (e.g. sulfonylureas, adenosine antagonists). The present review focuses on these different classes of drugs. Prudent use or avoidance of these drugs in patients who are at risk for myocardial infarction could theoretically limit ischemia and reperfusion injury.

INTRODUCTION

In the first part of this review on ischemic preconditioning, we described the infarct-size limiting effects of the naturally occurring phenomenon of ischemic preconditioning and the time-windows in which this effect occurs (228). Moreover, the interesting observation that a short period of ischemia also renders distant organs resistant to a subsequent prolonged period of ischemia was discussed. Finally, the most important triggers, mediators and end-effectors of ischemic preconditioning that have been identified so far were summarized. However, most data described in this part were derived from animal experiments. Because these studies have convincingly shown that ischemic preconditioning is the strongest form of *in vivo* protection against myocardial ischemic injury other than early reperfusion, the possibility to use this phenomenon in clinical practice would be very desirable. Despite state-of-the-art reperfusion strategies, 30-day mortality of myocardial infarction is still around 7 % (386). In addition, the prevalence of cardiac failure is rapidly increasing and is often caused by (ischemic) death of cardiomyocytes. Thus, there is need for additional therapeutic strategies that increase tolerance to ischemia and reperfusion injury. Exploitation of ischemic preconditioning may offer such a strategy.

To adequately exploit this mechanism in the everyday clinical setting, three more issues need to be addressed. First, the evidence that preconditioning also occurs in the human heart needs to be discussed. Secondly, if indeed protection can be seen in humans, could it be exploited to develop therapeutic strategies to protect the human heart against ischemic injury? In clinical practice, it is often not desirable or feasible to precondition myocardium with ischemia. Fortunately, the accumulating knowledge about the molecular mechanisms mediating preconditioning has provided us with the possibility to modulate ischemia and reperfusion injury pharmacologically, thus limiting infarct-size in the same way as ischemic preconditioning.. Finally, it is essential to identify those patients that may benefit from preconditioning and the situations in which preconditioning could be applied. In the present part of this review, we will consecutively discuss these three issues. Considering pharmacological preconditioning, special emphasis will be put on drugs that are used frequently in internal medicine.

DOES PRECONDITIONING OCCUR IN THE HUMAN HEART?

Analogous to the previously discussed animal studies, the evidence that ischemic preconditioning also occurs in humans has been derived from various experimental models, which are summarized in table 1. The most important difference between animal studies and human studies on this subject concerns the end-point that is used to estimate ischemic injury. Also, the design of the experimental protocol often differs substantially. In animal models, in contrast to human clinical practice, coronary occlusion if often induced and ended abruptly in otherwise healthy animals. Traditionally, in animal studies, the end-point is histological infarct size. For obvious reasons, this end-point cannot be used in human studies. Therefore, several models have been developed in which surrogate end-points are used to evaluate ischemic preconditioning in humans, recently reviewed by Tomai *et al* and Kloner *et al* (123, 273).

Table 1: Overview of the various *in vitro* and *in vivo* models of ischemic preconditioning in humans with main observations and limitations.

Method	Major end-points	Problems / Limitations
In vitro		
Cultured cardiomyocytes	Tryptan blue exclusion and lactate/LDH release	• Hypoxia instead of ischemia • Isolated cells, no infarct size
Isolated atrial trabeculae	Recovery of contractile function	• Hypoxia instead of ischemia • No direct measurement of cellular death • End-point determined by cell death and stunning
In vivo		
Warm-up phenomenon	Exercise tolerance	• Role of ischemic preconditioning as mediator controversial
Pre-infarction angina	Clinical outcome after myocardial infarction	• Confounded by more rapid thrombolysis
Repeated PTCA	ST-segment elevation, anginal pain, myocardial lactate extraction	• ST-segment change determined by $sarc_{KATP}$-channels • No direct measurement of cellular death • Possible collateral recruitment
Aortic clamping before CABG	Post-operative troponin/CK-MB release, post-operative recovery	• Confounded by peri-operative drugs, which affect preconditioning • No direct measure of cellular death
^{99m}Tc-Annexin A5 scintigraphy	Targeting of annexin A5	• Skeletal muscle instead of myocardium.

In vitro, classical as well as delayed preconditioning has been shown in cultured cardiomyocytes, using tryptan blue exclusion as end-point of simulated ischemia and reperfusion injury (387, 388). The existence of ischemic preconditioning has also been demonstrated in isolated human atrial trabeculae, obtained from patients undergoing heart surgery. In this model, using electrical field stimulation, recovery of contractile force after simulated ischemia and reperfusion is used as end-point (389). Later is was found that preconditioning in this model was also critically dependent on protein kinase C (PKC) activation and adenosine-tri-phosphate sensitive potassium channel (K_{ATP}-channel) opening and that adenosine A_1 and A_3 receptor stimulation could mimic preconditioning (390, 391).

These *in vitro* models are good candidates to screen drugs on their potential to mimic or modulate ischemic preconditioning, but cannot directly be extrapolated to clinical practice. In addition to these *in vitro* models, there are several observations in daily clinical practice that might be explained by ischemic preconditioning. The so-called warm-up phenomenon refers to the naturally occurring phenomenon, which is described in more than half of all patients with coronary artery disease, that performance is improved and ischemia-induced symptoms are attenuated during a second period of exercise compared with the first exercise test. Ischemic preconditioning has been suggested to be one of the possible causes of this phenomenon, particularly because the warm up phenomenon lasts no longer than 90 minutes (392). However, because adenosine receptor stimulation does not seem to be involved in warm up and because involvement of K_{ATP} channels is uncertain, a role for ischemic preconditioning in warm up remains controversial (reviewed in (392, 393)). Another naturally occurring phenomenon that could be explained by ischemic preconditioning is the possible infarct-size sparing effect of preinfarction angina. Many patients with acute myocardial infarction have experienced angina in the hours or days preceding the infarction. Several studies showed that indeed the presence of preinfarction angina, especially within 24 hours before infarction, is associated with improved clinical outcome after acute myocardial infarction, including death and the incidence of heart failure (394, 395), with reduced CK-release (394, 396) and with a smaller area of necrosis as assessed by nuclear imaging (397). Also, Solomon *et al* recently suggested that angina, reported during the three months preceding myocardial infarction, protects against left ventricular remodelling (398). However, not all studies showed this association (399). Moreover, Andreotti *et al* showed that preinfarction angina was associated with a more rapid reperfusion of the infarct related artery following thrombolysis, which is an attractive alternative explanation for the beneficial effect of angina (400). This finding is in accordance with the observations that preinfarction angina does only protect in patients treated with thrombolysis and not when treated with coronary angioplasty (401). In conclusion, although there is strong evidence that preinfarction angina renders the myocardium more resistant to a subsequent myocardial infarction, the role of ischemic preconditioning in this association remains controversial.

In addition to the abovementioned naturally occurring forms of preconditioning, there are also two models in which active interventions are able to trigger preconditioning and which are therefore better suited to effectively study the modulation of this protection by external factors such as drugs. First, in clinical practice, percutaneous transluminal coronary angioplasty (PTCA) offers the opportunity to electively and selectively apply ischemia to a well-defined myocardial region. In theory, the first coronary occlusion in a series of occlusions could increase resistance to subsequent

occlusions. Using this model, several studies showed that ST-segment shift on electrocardiography and subjective anginal pain were decreased during the second coronary occlusion, as well as wall motion abnormalities and lactate production (402-404), although some studies showed no protection (405, 406). Subsequently, the finding that the non-selective adenosine receptor antagonist aminophylline could block this protection (407) and that intracoronary infusion of adenosine (408) as well as bradykinin (409) followed by a short period of wash-out before the first inflation could mimic preconditioning further strengthened the probability that indeed ischemic preconditioning was responsible for the increased resistance to the second period of ischemia. However, these results have to be interpreted with caution because of two reasons. First, acute recruitment of collateral vessels is a major possible confounding factor (410, 411). Second, the most important surrogate end-point used in this model of preconditioning is ST-segment elevation on electrocardiography. However, ST-segment elevation is determined by opening of sarcolemmal K_{ATP}-channels (412), which, as outlined in the first part of this review, are probably not necessary for ischemic preconditioning to occur. Recently, it was clearly demonstrated that this parameter is not a good end-point of preconditioning by showing a dissociation between this parameter and infarct size limitation (413).

A second of the very few clinical scenarios in which cardiac ischemia is planned is coronary artery bypass grafting (CAGB). In this situation, ischemic preconditioning can by studied while avoiding the possible confounding of recruitment of collateral vessels by applying global cardiac ischemia instead of local ischemia. The evidence that ischemic preconditioning confers additional protection in CABG and the possible use of preconditioning in clinical practice has recently been comprehensively discussed (414, 415). Yellon's group were the first to show that pre-treatment with two 3-minute periods of cross-clamping and reperfusion before a 10-minute period of ischemia and ventricular fibrillation induced better preservation of left ventricular ATP-content and reduced post-operative troponin I release (125, 416, 417). Whether ischemic preconditioning is also able to confer additional protection to ischemia when other techniques than intermittent cross-clamp fibrillation are used, is more controversial. Illes *et al* found improvement in postoperative cardiac index and reduced requirement for inotropics with 1 minute aortic cross-clamping before cold blood cardioplegic arrest (418). Moreover, Lu *et al* found a reduction in postoperative CK-MB release and improved recovery of myocardial contractility in patients undergoing valve replacement with the use of cardioplegia (419). However, other groups were not able to demonstrate beneficial effects of ischemic preconditioning in the setting of cardioplegic arrest (420, 421). Considering pharmacological preconditioning, some studies have shown that pre-treatment with adenosine instead of short periods of ischemia and reperfusion before CABG was associated with better postoperative ventricular performance (422) and less CK-MB release (423), whereas others did not show a benefit from pre-treatment with a specific A_1-receptor agonist (125) or adenosine (424). The discordant results obtained with ischemic and pharmacological preconditioning in the setting of heart surgery could well be caused by two important possible confounders. First, in this setting anesthetics are always used concomitantly and as discussed in a later section, it is known that most anesthetics influence preconditioning in a positive or negative way. Moreover, there are indications that cardio-pulmonary bypass itself is able to precondition the myocardium, leaving little room for additional protection (425, 426).

Although the beneficial effect of ischemic preconditioning on the incidence of ischemia-reperfusion-induced arrhythmias remains controversial in animal models (427, 428), recent studies in man suggest clinical benefit. The incidence of ventricular tachyarrhythmias after declamping in CABG patients was shown to be significantly reduced by preconditioning with two 2-minute periods of ischemia and reperfusion (429).
In conclusion, there is a wealth of evidence that ischemic preconditioning also occurs in humans, but conclusive evidence and large-scale testing of the ability of drugs to mimic or inhibit preconditioning is still hampered by the lack of an optimal and easy-to-use human model. Ischemic preconditioning is not confined to cardiac tissue, but has also been described for liver, brain and skeletal muscle (430-432). Also, the mechanisms of ischemic preconditioning in heart and skeletal muscle share many similarities (433, 434) Recently, our group has developed and validated a new model of ischemic preconditioning in the forearm. Fundamental to this model is that ischemic exercise (isometric contraction of the finger flexors while the circulation is occluded with an upper-arm cuff) induces translocation of phosphatidylserines from the inside to the outside of cellular membranes of affected cells, which is considered an early marker of apoptosis. This process can be visualized by scintigraphic imaging of the arm and hand after injection of radiolabeled annexin A5, which selectively binds to these phosphatidylserine residues. With this model, we have shown that 10 minutes of forearm ischemia protects against increased annexine A5 binding induced by a subsequent 10-minutes of ischemic exercise, that infusion of adenosine into the brachial artery of the experimental arm mimics protection and that protection is inhibited by pretreatment with the adenosine receptor antagonist caffeine. By infusing target drugs into the brachial artery, it is easy to test their influence on ischemic preconditioning or ischemia-reperfusion injury *per se*. Apart from a research tool, this model may eventually be used in a clinical setting to individualize pharmacological strategies that are aimed to improve tolerance against ischemia and reperfusion.

PHARMACOLOGICAL PRECONDITIONING AND MODULATION OF ISCHEMIC PRECONDITIONING.

The elucidation, mostly from animal experiments, of great parts of the molecular machinery that is responsible for protection by ischemic preconditioning, has provided us with several rational targets for pharmacological intervention. Various drugs have been shown to be able to mimic ischemic preconditioning when applied instead of the preconditioning period of ischemia. On the contrary, several drugs also interfere with ischemic preconditioning and actually inhibit or reduce protection from ischemic preconditioning. An overview of drug classes that are able to influence preconditioning is provided in table 2. In this section, we preferentially discuss human studies. However, when these studies are unavailable, animal studies are used. It is important to realize that large interspecies differences exist with regard to preconditioning and the mechanism of preconditioning, and therefore, data derived from animal studies need to be interpreted with caution.

Table 2: Drugs with the ability to mimic or inhibit preconditioning. References preferentially indicate human studies; if not available, animal studies are referred to.

Mimicking preconditioning	Inhibition of preconditioning
Adenosine receptor agonists Adenosine (391, 408)	Adenosine receptor antagonists Theophylline, aminophylline, bamiphylline (389, 407, 435)
Nucleoside transport inhibitors *By increasing endogenous adenosine* Dipyridamole (436)	
K_{ATP} channel openers Nicorandil (437) Diazoxide (438)	K_{ATP} channel blockers Glibenclamide (438, 439)
Opioid agonists (440) Morphine (441)	Opioid receptor antagonists Naloxone (442)
α_1-adrenergic receptor agonists phenylephrine, norepinephrine (300, 371)	α_1-adrenergic receptor antagonists phentolamine (443).
β_1-adrenergic receptor agonists isoproterenol (444)	β_1-adrenergic receptor antagonists (445)
B2-bradykinin receptor agonists (409)	
ACE-inhibitors *By increasing bradykinin concentration* Captopril, lisinopril (446)	
Angiotensin II receptor antagonists Losartan (447)	
Volatile anesthetics Isoflurane, halothane, sevoflurane, enflurane, desflurane (448)	Intravenous anesthetics R-ketamine, thiopental and pentobarbital (449)
Nitric oxide donors Nitroglycerin (450)	
Statins Pravastatin (451)	
Ethanol (452)	
Corticosteroids (453)	COX-2 inhibitors *Inhibit only delayed preconditioning* High dose ASA (454); Celecoxib (361)

In this section we will highlight several drugs that are already used in daily clinical practice and which have the potential of mimicking or modulating preconditioning. Consecutively, nucleoside uptake inhibitors, ACE-inhibitors and AT1-receptor antagonists, HMG-CoA-reductase inhibitors, sulphonylureas, K_{ATP}-openers, anesthetics, and alcohol will be evaluated for their potential to

modulate ischemic preconditioning. Additionally, it will be discussed whether known positive or negative effects of these drugs on cardiovascular function or mortality could be explained by their preconditioning modulating effect.

Both animal and human studies have identified adenosine as one of the most important triggers of ischemic preconditioning. However, because of its very short elimination time, adenosine itself is not suited for administration to serve this goal. Moreover, more stable specific adenosine receptor agonists are not yet available for human use in clinical practice. However, by inhibiting the cellular uptake of endogenous adenosine, dipyridamole is able to increase the extracellular concentration of endogenous adenosine. Indeed, intravenous pre-treatment with dipyridamole significantly potentiated the infarct-size limiting effect of ischemic preconditioning in rabbit heart (191). In humans, intracoronary administration of dipyridamole before balloon inflation during PTCA also reduced anginal pain and ST-segment shift (455) and prevented deterioration of ventricular function during balloon occlusion (436). In clinical practice, efficacy of dipyridamole, given especially because of its presumed effect on platelet-aggregation, has long been subject of controversy. A recent meta-analysis concluded that in patients with vascular disease, there is no evidence that dipyridamole reduces the risk of vascular death, although in one study in patients after cerebral ischemia, dipyridamole reduced the risk of further vascular events (456). This lack of clinical benefit might be due to the fact that dipyridamole is not dosed high enough to adequately increase the endogenous adenosine concentration or because dipyridamole is often co-administered with acetylsalicylic acid, which might itself inhibit delayed ischemic preconditioning (454), offsetting the possible beneficial effects of dipyridamole.

Several studies showed that bradykinin is also involved as a trigger in ischemic preconditioning. In humans, bradykinin is able to mimic ischemic preconditioning in the model of repeated PTCA (409). Analogues to adenosine, direct bradykinin receptor agonists are not yet available for clinical human use. However, angiotensin-converting enzyme (ACE) inhibitors are known to inhibit the breakdown of bradykinin, thus increasing the concentration of endogenous bradykinin (figure 1) (457). Considering preconditioning of the myocardium, animal studies have demonstrated that pre-treatment with ACE inhibitors reduces infarct size (458), potentiates the acute (459) as well as delayed (460) infarct size limiting effect of subthreshhold ischemic stimuli and attenuates myocardial stunning (461). Moreover, selective bradykinin B2-receptor antagonists inhibit these beneficial effects of ACE inhibitors (459, 461). Similar results were obtained in human atrial trabeculae, obtained during CABG, in which post-ischemic recovery of contractile function was significantly increased by pre-treatment with captopril and lisinopril in combination with a subthreshold stimulus for ischemic preconditioning. These beneficial effects were again completely prevented by a specific bradykinin B2 receptor antagonist (446). These potentiating effects of ACE inhibitors on ischemic preconditioning could be one of the mechanisms responsible for the favourable effects of these drugs on cardiovascular death and the incidence of heart failure in several clinical trial, e.g. the HOPE trial (462). Surprisingly, AT1-receptor antagonists, initially presumed not to influence the kallikrein-kinin system, could also limit infarct size in rat (447) and pig (458) hearts and intriguingly, this effect could also be blocked by bradykinin antagonists (447). This observation is in contradiction with earlier studies, showing inhibitory effects of AT1-receptor

antagonism on the effect of ischemic preconditioning (463). One explanation for this beneficial effect of AT1-receptor antagonists could be that during blockade of the AT1-receptor, AT2-receptor stimulation by angiotensin II is enhanced (Figure 1).

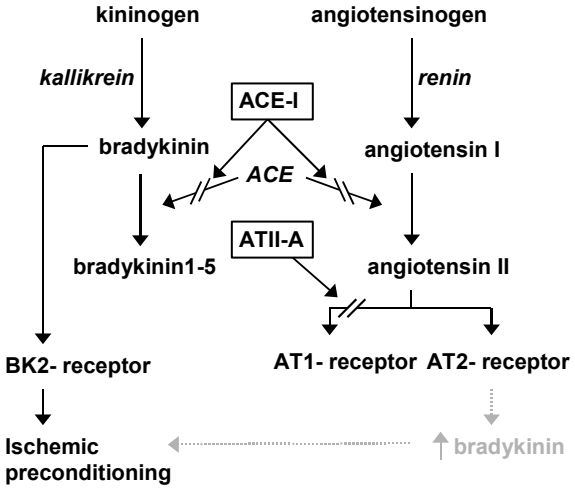

Figure 1: Schematic illustration of the interaction between the renin-angiotensin and the kallikrein-kinin system and the effects of angiotensin converting enzyme inhibitors (ACE-I) and angiotensin II antagonists (ATII-A). This illustration shows how these drugs mimic ischemic preconditioning. BK = bradykinin. ACE-I inhibit breakdown of bradykinin, which stimulates bradykinin BK2 receptors. ATII-A only block the AT1-subtype receptor. Subsequent increased stimulation of the AT2-receptor by endogenous angiotensin II could activate the kallikrein-kinin system, also leading to an increased release of bradykinin.

AT2 receptor stimulation has recently been shown to activate the kallikrein-kinin system and thereby stimulate bradykinin release (464). Indeed, it was subsequently shown that the vascular effects of candesartan were blocked by bradykinin antagonism (465). This effect might explain the similar effects on mortality of ACE inhibitors and ATII-antagonists in patients who are at high risk for cardiovascular events after acute myocardial infarction (466). It needs to be emphasized, though, that it is very difficult to investigate the preconditioning-mimicking effect of drugs in large clinical trials, because preconditioning would not influence the incidence of cardiovascular events, but rather the outcome, once an event has occurred.

3-Hydroxy-3-methylglutaryl (HMG)-Co-enzyme A (CoA) reductase inhibitors form another class of drugs, widely prescribed in cardiovascular compromised patients, which have been suggested to protect against ischemia-reperfusion injury. The beneficial effects of HMG-Co-A-reductase inhibitors on cardiovascular morbidity and mortality in patients at risk for cardiovascular disease are widely appreciated. Beyond their ability to halt the process of atherosclerosis, mimicking of ischemic preconditioning has also been suggested to contribute to these major beneficial effects. Ueda *et al* showed that the infarct-size limiting effect of ischemic preconditioning was blunted in

hypercholesterolemic rabbits and that pravastatin, added to their diet, completely restored this, without affecting plasma total cholesterol, HDL and triglycerides (451). This was explained by the finding that pravastatin also restored the activation of the enzyme ecto-5'-nucleotidase during the preconditioning ischemia, which was attenuated in the untreated hypercholesterolemic rabbits. As ecto-5'-nucleotidase converts adenosine-mono-phosphate into adenosine, this could well contribute to the observed effect. Later it was shown that lovastatin and simvastatin also enhanced ecto-5'-nucleotidase activity *in vitro* (467). In the recent study by Lee *et al*, hyperlipidemic patients with coronary artery disease were randomized to pravastatin or placebo for 3 months before PTCA. Patients on pravastatin had less ST-segment shift, anginal pain and myocardial lactate production during the first balloon occlusion than the control group and this protection was abolished by pre-treatment with the adenosine-receptor antagonist aminophylline, suggesting that the cardioprotection offered by pravastatine was mediated by adenosine (468). However, the treated patient group also had significantly lower plasma cholesterol levels. Because of these results, it is attractive to speculate that stimulation of ecto-5'-nucleotidase could be one of the mechanism that mediate the well-known protection of statins on the cardiovascular system. However, it needs to be stresses that other mechanisms of protection by this class of drugs might be present. Bell *et al* recently showed very elegantly that in mice hearts, the administration of atorvastatin during reperfusion after a period of ischemia significantly reduced infarct size independent of lipid lowering (469). This protection was achieved by activation of a signalling cascade involving phosphatidyl inositol 3-kinase (PI3K), the protein kinase Akt and eNOS. An alternative mechanism of cardioprotection by statins include inhibition of neutrophil activation and preservation of NO-synthase activity after ischemia and reperfusion (for review (470)), which could result from inhibition of the mevalonate pathway and subsequent inhibition of the Rho/Rho kinase pathway (471).

The last drug that has been shown to mimic preconditioning in humans *in vivo* and that acts on the level of the triggers of ischemic preconditioning is the NO donor nitroglycerin. NO has been implicated especially in delayed preconditioning and this has been tested recently in the model of repeated PTCA (450). Patients admitted for stable or unstable angina were randomized to receive a 4-hour intravenous infusion of nitroglycerin or placebo 24 hours before PTCA. It appeared that nitroglycerin pre-treatment, independent of collateral recruitment, rendered the heart resistant against ischemia, as assessed by ST-segment shift, wall motion and subjective pain.

More distal to the trigger phase of ischemic preconditioning, opening of mitochondrial K_{ATP}-channels is essential for the occurrence of protection by ischemic preconditioning. Drugs that interfere with K_{ATP}-channel opening could therefore theoretically inhibit this protection. Indeed, using recovery of contractile function of human atrial trabeculae as end-point of ischemic injury, Cleveland *et al* showed in an observational study that preconditioning was abolished in patients with type 2 diabetes using glibenclamide or glipizide compared with type 2 diabetics on insulin (472). Moreover, it was shown that pretreatment with 10 mg of glibenclamide orally before PTCA abolishes ischemic preconditiong as assessed by anginal pain and ST-segment shift in non-diabetics (439) and in the same model, ischemic preconditioning was inhibited in type 2 diabetics who were chronically on glibenclamide (473). Interestingly, in the same model, the newer sulfonylurea

glimepiride did not abolish protection by ischemic preconditioning (473, 474), possibly because it would block extra-pancreatic K_{ATP}-channels to a lesser extent than glibenclamide. Finally, Scognamiglio *et a*l showed that type 2 diabetics randomized to the use of insulin had less myocardial dysfunction during dipyridamole stress-echocardiography than patients on glibenclamide (475). However, this model is not well suited for this purpose, because dipyridamole itself is able to provide cardioprotection, as mentioned earlier. Despite the limitations inherent to the human models used, glibenclamide does seem to inhibit ischemic preconditioning. Does this mean that diabetics who use sulfonylureas are at increased risk for cardiovascular morbidity and mortality? This discussion was opened by the observation in the UGDP study that patients on tolbutamide had an increased cardiovascular mortality rate (476). In the UKPDS, treatment with metformin decreased mortality, whereas treatment with glibenclamide did not reduce mortality (477, 478). Additionally, various smaller trials provided conflicting results on the effect of sulfonylureas on cardiovascular morbidity and mortality (479). In conclusion, there is no convincing evidence that use of sulfonylureas is associated with worse cardiovascular outcome in general clinical practice. Interestingly, in special situations of profound cardiac ischemia, sulfonylureas may have detrimental effects: diabetics on sulfonylureas did have a higher in-hospital mortality after PTCA for acute myocardial infarction compared to diabetics not on sulfonylureas (480). However, because most of these latter patients were on insulin, this could also point to a beneficial effect of insulin. At this point, it needs to be realized that insulin, independent of glucose, can reduce myocardial infarction, when administrated early in reperfusion, acting via the Akt prosurvival pathway (481, 482).

In contrast to K_{ATP}-channel blocking, pharmacological opening of these channels could provide beneficial effects on myocardial ischemia-reperfusion injury. Indeed, many animal studies showed that pre-treatment with the K_{ATP}-channel opener diazoxide mimics the infarct-size limiting effect of ischemic preconditioning (317). Similarly, ischemic preconditioning mimicking effects of diazoxide have been shown in the human atrial trabeculae model (438, 483). Very recently, Wang *et al* demonstrated that patients randomized to pretreatment with an intravenous bolus of diazoxide 5 minutes before commencing cardiopulmonary bypass for CABG have significantly better improvement of cardiac index after surgery (484). More clinically oriented research has been done with nicorandil, a hybrid of a nitrate and a K_{ATP}-channel opener, registered for the use in patients with stable angina. This drug has been shown to reduce infarct size in several animal models via its opening of K_{ATP}-channels, both acutely and after 24 hours (485, 486). In humans, pre-treatment with an intravenous bolus of nicorandil before PTCA in patients with stable angina appeared to limit ST-segment shift independent of myocardial blood flow (487, 488). However, it needs to be emphasized that ST-segment shift is determined by sarcolemmal K_{ATP} channels, which are influenced by nicorandil but probably are less important in preconditioning, making this parameter highly unreliable for this goal. That these beneficial effects of nicorandil in the experimental setting could indeed also apply to the clinical setting, is demonstrated by Patel *et al* (489). They showed that patients with unstable angina who were randomized to nicorandil 20 mg orally twice daily added to an aggressive anti-anginal treatment with acetylsalicylic acid, beta-blockers and diltiazem, suffer less myocardial ischemia and ventricular arrhythmias in the first 48 hours after admission than the control group. The preconditioning mimicking effect of nicorandil could also have

contributed to the results of the Impact Of Nicorandil in Angina (IONA) study, which showed that nicorandil, added to conventional anti-anginal therapy, induces a significant reduction in major coronary events in patients with stable angina and additional cardiovascular risk factors (490). The role of preconditioning in this study, however, remains speculative.

Another class of drugs that is known for the potential to influence K_{ATP}-channels, are volatile anesthetics. Because of the inherent timing before start of operations and because of the relative ease of administration, this group of drugs would be especially suited to use for early cardioprotection. Indeed, in 1997 it was first described that isoflurane mimics the protective effect of ischemic preconditioning in rabbits and dogs (491, 492). Today, it is known that most anesthetics are able to mimic, enhance or inhibit ischemic preconditioning, which is recently reviewed by Zaugg *et al* (448, 493) and Riess *et al* (494). In animal studies, protective effects have been demonstrated for isoflurane, enflurane, halothane, sevoflurane and desflurane. Adenosine A_1-receptor stimulation, PKC activation and opening of K_{ATP}-channels all have been implicated in the mechanism of this protection (493). On the contrary, various intravenous anesthetics have been shown to inhibit opening of mitoK_{ATP}-channels *in vitro* and it was demonstrated that R-ketamine, thiopental and pentobarbital inhibit diazoxide-induced protection in isolated rat ventricular myocytes (448, 449). Recently, a few small trials have investigated the effects of isoflurane, enflurane and sevoflurane preconditioning in patients undergoing CABG. These data provide evidence, although not always significant, that these anesthetics are able to provide some protection as assessed by post-operative CK-MB and troponin I release and post-operative myocardial function (448). A recent randomized study even concluded that sevoflurane preconditioning in CABG patients preserves myocardial as well as renal function as assessed by post-operative plasma levels of N-terminal pro-brain natriuretic peptide and cystatin C, respectively. However, more traditional markers (CK-MB, troponin T and creatinine) were not improved by preconditioning (495). Finally, considering anesthesia, it has to be mentioned that opioid receptor agonists, which are frequently used in the peri-operative timeframe, are also able to provide cardioprotection by preconditioning in animal models (441) and in isolated human atrial trabeculae (440). Using the model of repeated PTCA in humans *in vivo*, Tomai *et al* showed that pre-treatment with the opioid-receptor antagonist naloxone completely blocks the protective effect of ischemic preconditioning (442). Interestingly, recently it was shown that volatile anesthetics and opioids may work in conjunction to confer protection against myocardial infarction through potentiation of cardiac K_{ATP}-channel opening (496).

Besides pharmacological agents, compounds present in daily food and drink could also be able to provide protection against ischemia-reperfusion. It is known that moderate alcohol consumption is associated with a decreased risk of cardiovascular disease (497, 498). Moreover, it was found that moderate drinking was associated with increased survival once acute myocardial infarction had occurred (499). Besides beneficial alterations in lipid metabolism and platelet function, preconditioning of the myocardium by ethanol could contribute to this beneficial effect of alcohol consumption. Indeed, accumulating evidence from various animal models demonstrates that chronic as well as acute ethanol consumption reduces myocardial ischemia-reperfusion damage by mimicking ischemic preconditioning (452, 500-503). Hearts from guinea pigs drinking ethanol for 3-

12 weeks showed improved functional recovery and reduced myocyte damage after ischemia and reperfusion (452). This preconditioning mimicking effect was completely abolished by adenosine A_1 receptor blockade during the index ischemia (452). Indeed, it was shown earlier that ethanol increases extracellular adenosine concentration by inhibiting cellular adenosine uptake (504), and this mechanism could be involved in the previously described beneficial effect of ethanol. However, in rats, alcohol-induced cardioprotection was not blocked by adenosine receptor antagonists, whereas α-adrenergic antagonism did block this protection, suggesting species specific signalling (500). More recently, Miyamae *et al* showed that chronic ethanol consumption induces a sustained translocation of PKC-ε from the cytosolic to the particulate fraction and that cardioprotection by ethanol was critically dependent on PKC activity during the index ischemia (501). Acute ethanol ingestion shortly before the ischemic insult, resulting in a concentration similar to that achieved after 1-2 alcoholic beverages, similarly provided protection by direct activation of PKC-ε (503). Finally, the infarct-size limiting effect of chronic ethanol ingestion in dogs was abolished by administration of glibenclamide during ischemia, thus providing evidence that opening of K_{ATP}-channels is crucial for this protection to occur (502). In conclusion, chronic as well as acute consumption of alcohol provides protection against ischemic injury in several animal species via adenosine and α-adrenergic receptor stimulation, PKC-ε translocation and opening of K_{ATP}-channels.

THERAPEUTIC EXPLOITATION OF ISCHEMIC OR PHARMACOLOGICAL PRECONDITIONING

From the evidence outlined in the second part of the present paper, it appears that also in the human myocardium, ischemic preconditioning can significantly increase tolerance to ischemia and reperfusion. However, in clinical practice, the application of short periods of ischemia to induce preconditioning is in most circumstances not desirable or feasible. However, several classes of drugs have been described with the potential to enhance, mimic or inhibit ischemic preconditioning. The prudent use, or avoidance, of these agents may be a more benign approach to elicit cardioprotection in clinical practice.

Because of the relatively tight time boundaries of protection by ischemic and pharmacological preconditioning, it is essential to apply the pharmacological intervention shortly before the prolonged ischemic period. Unfortunately, myocardial ischemia is seldom planned and accurately predicted. However, two situations in which temporary myocardial ischemia can readily be predicted are PTCA and CABG.

Although routine PTCA carries a small risk for complications, this risk is increased in high-risk situation, such as unstable angina. Especially in these situations, pre-treatment with preconditioning-mimicking drugs could be beneficial. Conversely, the temporary withdrawal of drugs that are known to interfere with preconditioning, such as K_{ATP}-blockers or adenosine antagonists, could increase tolerance to ischemia. Garratt *et al* have shown in an observational study that diabetics using sulfonylureas have higher in-hospital mortality after PTCA for acute myocardial infarction than diabetics who do not use sulfonylureas (480). Again, it needs to be realised that this survival benefit could also be caused by the beneficial effect of insulin in the control group. Interestingly, in the setting of PTCA, preconditioning by repeated balloon inflations could also be used to stratify patients for their risk of adverse ischemic events. Recently, Laskey *et al* showed that 20% of patients undergoing PTCA failed to manifest ischemic preconditioning, and

that this was significantly associated with an increased risk of death or non-fatal myocardial infarction at one year of follow-up (505).
A second situation in which cardiac ischemia is planned and may consequently be preceded by a preconditioning stimulus is CABG, as described above. However, the protective effect of ischemic or pharmacological preconditioning is still controversial, especially when other techniques than intermittent cross-clamp fibrillation are used (414, 415). It is argued that the protection afforded by cardioplegia and anesthetics leaves little room for additional protection by preconditioning (414, 415). Moreover, it has been shown that cardiopulmonary bypass itself is able to provide cardioprotection comparable to classis ischemic preconditioning in sheep hearts (425). Similarly, in a recent study in humans, preconditioning with ischemia only offered additional protection during CABG when no cardiopulmonary bypass was used (426). Thus, preconditioning may only be indicated in settings in which conventional protection is anticipated to be suboptimal, e.g in long duration or severe atherosclerosis (415). Moreover, when considering protection to postoperative pump failure, it needs to be realized that stunning, more than discrete necrosis or apoptosis, might be responsible for this, and that early preconditioning probably does not protect against stunning. Perhaps a more successful, albeit less heroic, approach might be the elimination before surgery of factors with potential inhibiting effects on preconditioning, such as the use of sulfonylureas or caffeine.
Finally, considering anticipated periods of cardiac ischemia, preliminary evidence exists that ischemic preconditioning might be beneficial in transplantation. In sheep heart, recovery of systolic function was improved when a short period of ischemia was applied before the explantation (506).

Perhaps more benefit from pharmacological preconditioning could be expected when applied to patients at high risk for myocardial infarction, despite adequate conventional treatment. This would particularly concern patients with non-ST-segment acute coronary syndromes, including unstable angina, who are at high risk of progression to complete coronary occlusion. More than 10% will die or suffer a myocardial infarction within 6 months, with half of these events occurring in the acute phase (507). Pharmacological preconditioning during this phase could potentially reduce the amount of ischemic damage. However, as the duration of protection afforded is limited, repeated dosing of the preconditioning drug is necessary to maintain the preconditioned state. Although a 72-hour continuous infusion of an adenosine A_1-selective agonist in rabbits was not able to limit infarct-size, suggesting receptor down-regulation (508), a more recent study in rabbits showed that repeated bolus injections of an adenosine A_1-selective agonist at 48-hour intervals still provides strong limitation of infarct-size at day 10 (509). Moreover, consumption of dipyridamole, added to the drinking water for 2-6 weeks, resulted in an attenuation of ischemia-reperfusion injury in guinea pigs (510).
Even more benefit from pharmacological modulation of preconditioning might be expected in large groups of patients with an increased baseline risk for cardiovascular disease, such as diabetics. Sulfonylureas are associated with an unexpected and unexplained small increase in cardiovascular mortality in several trials, as described previously. Reducing the use of sulfonylureas could potentially confer benefit to this patient group, with regard to cardiovascular morbidity and mortality.

Very recently, several studies showed that pharmacological interventions during early reperfusion are also able to limit infarct-size (reviewed in (346)). This approach circumvents the problem that the ischemic insult is mostly unpredictable, because these drugs can be given at reperfusion rather than before the event and is therefore more clinically applicable, but outside the scope of this review on preconditioning. Briefly, in the AMISTAD trial, it was shown that adenosine as an adjunct to thrombolysis, results in a significant reduction in infarct size (511). Also, infarct-size limitation has been shown for insulin (481, 482), atorvastatin (469), 5'-(N-ethylcarboxamido) adenosine and bradykinin (512), all via activation of the PI3K/Akt pathway during reperfusion. Also, cyclosporine limits infarct-size when administered during reperfusion by inhibiting opening of the MPTP (513). Additional studies have to be performed to show whether this approach could offer clinical benefits.

A final word of caution regarding the potential therapeutic benefits of preconditioning concerns the reported effects of ageing and disease on ischemic preconditioning. In the literature, it is repeatedly mentioned that the protective effect of preconditioning may be lost in aged myocardium, in which cardioprotection is undoubtedly more relevant, although there is still no consensus on this subject. Most studies on isolated hearts show that the effect of preconditioning is decreased in aged rats (514-517), but not in aged rabbits (518, 519). In humans, a similar controversy exists in the various models of ischemic preconditioning (520-523). Decrease in norepinephrine release during the preconditioning episode (514), attenuated activation of K_{ATP}-channels (521) and failure of adequate translocation of PKC isoforms (516) all have been implicated in this reduced protective effect of preconditioning in the aged heart.
A similar controversy exists as to whether protection by preconditioning is still present in the diseased heart, especially concerning diabetes and hypercholesterolemia, the very conditions in which cardioprotection is particularly important. Although some studies indeed showed protection by ischemic preconditioning in diabetic rats (524), most studies in rabbits and dogs demonstrated that diabetes abolishes protection by ischemic preconditioning (525-527). In dogs, it appeared that both streptozotocin-induced diabetes and hyperglycemia by dextrose-infusion inhibit the infarct-sparing effect of preconditioning, probably due to impaired activation of mitoK_{ATP}-channels (526, 528, 529). Similarly, in an observational study both preconditioning by ischemia and by pre-treatment with diazoxide was abolished in atrial tissue taken from patients with type 1 diabetes using insulin and from patients with type 2 diabetes using sulfonylureas but it was not abolished in patients with diet controlled diabetes (530). Finally, it is reported in the literature that the protective effect of pre-infarction angina is diminished in patients with diabetes (398, 531). The diminished protection afforded by ischemic preconditioning in patients with diabetes could well contribute to the consistently shown worse outcome after myocardial infarction in these patients compared to patients without diabetes (532, 533). Considering hypercholesterolemia, there is less evidence from the literature. There are studies that show preserved protective effects of ischemic preconditioning (534, 535) as well as studies that show reduced protection by ischemic preconditioning in hypercholesterolemic rabbits (451). Considering evidence in humans, recently it was shown that in patients with high plasma cholesterol, the preconditioning by repeated PTCA was reduced as compared to patients with normal cholesterol levels (536). With regard to other other risk factors for atherosclerosis, little is known about the influence of smoking, hypertension, and

hyperhomocysteinemia on the effect of ischemic preconditioning. Regarding hypertension, it was shown that protection was still present in spontaneously hypertensive rats and in hypertrophied myocardium from saline loaded rats (537, 538).
When interpreting these data on aged and diseased hearts, one has to bear in mind that the exact signalling mechanism involved in preconditioning is dependent on the nature of the preconditioning stimulus (297). Because the effects of aging or disease might be limited to specific triggers, such as adenosine (539), it is conceivable that failure to precondition these hearts is influenced by the choice of the preconditioning stimulus.

In summary, there is a wealth of both *in vitro* and *in vivo* evidence that ischemic preconditioning also occurs in humans. Since the description of this phenomenon, several classes of drugs have been described which are able to mimic, enhance or inhibit ischemic preconditioning. The use or avoidance of these drugs before procedures known to induce myocardial ischemia or in patients at risk for myocardial infarction in general could theoretically reduce ischemia and reperfusion injury and improve outcome. We recently developed a minimally invasive technique to monitor ischemic tolerance in humans *in vivo*. Future clinical trials with this technique are needed to address the question whether this method can be used to individualize pharmacotherapy in order to optimize resistance to ischemia-reperfusion and outcome in patients who are particularly vulnerable to ischemic cell death: patients at risk for arterial thrombosis and patients with heart failure.

ACKNOWLEDGEMENTS
N.P. Riksen is a MD-clinical research trainee financially supported by the Netherlands Organization of Scientific Research (ZonMw). The contribution of G.A. Rongen has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences (KNAW).

CHAPTER 4.3

ANNEXIN A5 SCINTIGRAPHY OF FOREARM AS A NOVEL *IN VIVO* MODEL OF SKELETAL MUSCLE PRECONDITIONING IN HUMANS

Circulation 2005;111:173-8

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ABSTRACT

Background: Nonlethal ischemia and reperfusion reduces ischemia-reperfusion-induced cell death, a phenomenon called ischemic preconditioning. In animal models, this potent endogenous protection is mimicked *in vivo* by administration of adenosine. In humans, exploitation of ischemic preconditioning is hindered by the lack of an appropriate *in vivo* model to study this phenomenon. To solve this problem, we aimed to set up an easy-to-use human *in vivo* model to study ischemic or pharmacological preconditioning.

Methods and Results: Healthy male volunteers performed unilateral ischemic handgrip. At reperfusion, we intravenously injected Tc-99m labeled annexin A5, a presumed marker of ischemic injury, and we imaged both forearms and hands simultaneously with a gamma camera. Region of interest analysis (counts per pixel) and subsequent calculation of the percentage difference in radioactivity between experimental and control hands (thenar muscle; mean±SE) revealed significant uptake to the ischemically exercised tissue (26±3% at four hours after reperfusion; $P<0.05$). This selective localization of annexin A5 was reduced by ischemic preconditoning (10 minutes of ischemia plus reperfusion prior to ischemic exercise) or by infusion of adenosine into the brachial artery to 6±1% and 10±3% respectively ($P<0.05$ versus ischemic exercise alone), resembling observations in animal models with infarct size as en endpoint. Appropriate control experiments supported our conclusion.

Conclusions: Annexin A5 scintigraphy can be applied to test pharmacological or physiological interventions for their ability to prevent ischemia-reperfusion injury.

Keywords: adenosine, exercise, ischemia, scintigraphy

INTRODUCTION

Ischemic preconditioning is defined as increased tolerance against ischemia-reperfusion injury resulting from a previous short exposure to ischemia (122). Since the original description of this phenomenon by Murry *et al* in 1986 in the canine heart, the underlying mechanism of action has partially been revealed (272). Ischemia-induced release of adenosine activates adenosine receptors (A_1 and A_3) on cardiomyocytes, followed by activation of protein kinase C, ultimately resulting in opening of mitochondrial ATP-sensitive potassium channels. Influx of potassium and depolarization of the mitochondrial inner membrane delays cell death during subsequent periods of ischemia, probably by inducing a small increase in mitochondrial volume (336). Ischemic preconditioning reduces the release of mitochondrial cytochrome C and prevents apoptosis (278, 343, 540). Whether this explains the full protective action of ischemic preconditioning is not known. Apart from the heart, other organs such as liver, brain and skeletal muscle are protected by ischemic preconditoning in various species such as rat, pig, dog and rabbit (430-432). These findings suggest a universal endogenous protective phenomenon against the deleterious sequelae of ischemia.

The mechanism underlying ischemic preconditioning suggested the possibility of pharmacological modulation of cellular tolerance to ischemia-reperfusion. In animal models various drugs, that are currently used in clinical practice to prevent or treat acute ischemic events interfere with ischemic preconditioning or modulate ischemia-reperfusion injury. For example, adenosine and opiates mimic ischemic preconditioning ('pharmacological preconditioning'), (441, 541) whereas ATP-sensitive potassium channel blockers and adenosine receptor antagonists inhibit ischemic preconditioning (542). Insulin and statins reduce ischemia-reperfusion injury by a mechanism that is, at least partially, independent from the signal transduction pathway of ischemic preconditioning (451, 543). Knowledge of the effect of (cardiovascular) drugs on ischemia-reperfusion injury will increase rational pharmacotherapy in patients who are at risk for ischemic events. Unfortunately, translation of these observations from animals to humans is hindered by the lack of a specific model to study ischemia-reperfusion injury in conscious humans *in vivo* (123).

Recently, annexin A5 scintigraphy has been developed as a method to detect early signs of cell death in humans *in vivo* (137, 544). Annexin A5 is an endogenous protein that binds with high affinity to negatively charged phosphatidylserine (545). Due to an active translocase, phosphatidylserine is located almost exclusively on the inner leaflet of the lipid bilayer of the normal cell membrane. Early in the process of apoptosis, the asymmetric distribution of phosphatidylserine is lost and phosphatidylserine is exposed on the outer surface of the cell, thus providing binding sites for extracellular annexin A5 (546). In mice, 90 minutes of hindlimb ischemia followed by a 3 hour period of reperfusion induced apoptosis of vascular cells and skeletal muscle fibers, which was reduced by a caspase inhibitor (547). To our knowledge, apoptosis has not been studied after shorter bouts of ischemia in combination with exercise. After mild ischemia, reversible phosphatidylserine externalization has recently been reported without subsequent development of cell death (548). Therefore, we hypothesize that the combination of a relatively short period of exercise during local blockade of the circulation induces externalization of phosphatidylserine and binding of annexin A5 which can be used to detect early and reversible ischemia-reperfusion injury.

The aim of this study was to validate annexin A5 scintigraphy as a tool to detect ischemia-reperfusion injury in humans *in vivo*. We reasoned that a relevant marker of ischemia-reperfusion injury should also be able to detect the protective effect of ischemic and pharmacological preconditioning with adenosine. We selected forearm skeletal muscle for three reasons. First, after intravenous administration of annexin A5, the non-ischemic forearm could serve as an internal control to subtract non-specific background activity that results from circulating radiotracer. Second, a validated model to detect ischemia-reperfusion injury of forearm skeletal muscle would allow efficient pharmacological screening by infusion into the brachial artery of drugs that could potentially modulate tolerance to ischemia-reperfusion injury. Finally, forearm ischemic load is well defined by occlusion of the forearm circulation without confounding by collateral circulation.
This study addresses three main questions: does ischemic isometric muscle exercise induce local targeting of radiolabeled annexin A5, can this targeting be prevented by a previous bout of ischemia and reperfusion (ischemic preconditioning) and finally, does local infusion of adenosine protect against subsequent injury from ischemic exercise?

METHODS

Subjects

After informed consent, a total of 43 healthy male volunteers (age 18-34 years) participated in six separate experiments. Subjects with cardiovascular disease, hypertension (systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg, measured sphygmomanometrically in supine rest) or diabetes mellitus (fasting glucose > 7.0 mmol/L or random glucose > 11.0 mmol/L) were excluded. Volunteers were free of medications and they were asked to abstain from caffeine-containing beverages for at least 24 hours before the start of the experiment. The protocol was approved by the Institutional Review Board of the UMC Nijmegen.

General procedures

Volunteers were studied in sitting position after cannulation of an antecubital vein of the dominant arm for injection of radiolabeled annexin A5 (see below). Maximal voluntary contraction was determined in the non-dominant arm with a handgrip dynamometer (Baseline Hydrolic Hand Dynamometer, Fabrication Enterprise Inc., Irvington, New York, USA).

Ischemic exercise

In 10 volunteers, the circulation of the non-dominant forearm was occluded for 10 minutes using an upper arm cuff which was inflated to 200 mmHg (ischemic exercise, Isch Ex). Immediately after occlusion of the forearm circulation, ischemia was combined with isometric contractions of the finger flexors at 50% of maximum voluntary contraction. These contractions were performed rhythmically: 5-second contraction followed by 5-second relaxation until the volunteer was exhausted. The total duration of ischemia was 10 minutes independent from the duration of contractions. Radiolabeled annexin A5 (0.1 mg protein, 500 MBq Tc-99m) was administered intravenously immediately after the start of reperfusion. Forearms were imaged at 0, 1, 2, and 4 hours post-injection. For this purpose, flexor muscles of both forearms were positioned on the gamma camara (Siemens Orbiter, Hoffman Estates, Illinois, USA, equipped with low-energy high resolution collimators) in pronated position and scanned simultaneously until at least 100000

counts were detected. After imaging of the flexor muscles, the palmar sides of both hands were placed on the gamma camera and images of at least 50000 counts were detected.

Ischemic preconditioning followed by ischemic exercise

In a separate group of 8 volunteers the ischemic exercise was preceded by a period of 10 minutes of forearm ischemia and 10 minutes reperfusion without simultaneous exercise (IP+Isch.Ex.). Pilot studies (data not shown) indicated that this period of ischemia without concomitant exercise did not induce targeting of annexin A5. Immediately after ischemic exercise, annexin A5 was infused and scintigraphic images were performed as described for the Isch.Ex. group.

Adenosine followed by ischemic exercise

In a third group of 9 volunteers the brachial artery was cannulated at least 30 minutes prior to drug infusion. In this group, adenosine (50 µg/min/dl forearm tissue; Adenocor®, Sanofi-Synthelabo Inc., Maassluis, the Netherlands) was infused into the brachial artery for 10 minutes, beginning 20 minutes before the start of the ischemic exercise (ADO+Isch.Ex.). Immediately after ischemic exercise, annexin A5 was infused and scintigraphic images were performed as described for the Isch.Ex. group. In this group, the measurement at 2 hours after reperfusion was omitted.

Phentolamine followed by ischemic exercise

In a separate group of 10 volunteers, phentolamine (15 µg/min/dl forearm tissue; Regitine®, Novartis Pharma Inc., Arnhem, the Netherlands) was infused instead of adenosine to study the effect of vasodilation *per se* on subsequent ischemia-reperfusion injury (PHENT+Isch.Ex.). In this group, the measurements at 0 and 2 hours after reperfusion were omitted for logistical reasons. Otherwise, the same protocol was performed as in the ADO+Isch.Ex. group.

Uptake of technetium-99m-labeled albumin after ischemic exercise

In an additional group of three volunteers, nonspecific mechanisms of targeting, such as changes in capillary permeability or blood flow were explored. For this purpose, these individuals performed ischemic exercise as described above. Instead of annexin A5, an equimolar dose of Tc-99m-labeled albumin was injected immediately upon reperfusion. Subsequently, the forearms were scanned at 1 and 4 hours after reperfusion as described above.

Delayed injection of annexin A5 after ischemic exercise

Finally, the influence of timing of the annexin A5 injection was studied in three volunteers. This group performed ischemic exercise as described above. In this group, annexin A5 (0.1 mg protein, 500 MBq Tc-99m) was injected 1 hour after reperfusion instead of immediately upon reperfusion. Both forearms were scanned immediately and 3 hours after injection of annexin A5 (1 and 4 hours after reperfusion) as described above.

Preparation of radiopharmaceuticals

Radiolabeled annexin A5 was freshly prepared before each experiment by adding Tc-99m Pertechnetate (1500 MBq) in the presence of stannous tricine to succinimidylhydrazinonicotinamide (HYNIC)-conjugated recombinant human annexin A5 (NAS 2020, 0.275 mg per vial, Theseus

Imaging Corporation, Boston, Massachusets, USA). Radiochemical purity as checked by ITLC was always higher than 95%. The labeled annexin A5 solution was filtered with a low protein binding 0.22 µm filter (Millex GV, Millipore #SLGV-0.25 BS). Finally, 500 MBq of the labeled annexin A5 (0.1 mg) was further diluted with NaCl 0.9% and immediately administered to the volunteer.
Radiolabeled albumin was prepared analogous to radiolabeled annexin A5. Human serum albumin (200 mg/ml, Cealb, Sanquin CLB, Amsterdam) was conjugated with HYNIC and stored in vials containing 0.6 mg HYNIC-conjugated albumin (approximately equimolar amount of protein compared with the annexin A5 vials). Radiolabeled albumin was freshly prepared before each experiment by adding Tc-99m Pertechnetate (1500 MBq) in the presence of stannous tricine to a vial. Radiochemical purity as checked by ITLC was always higher than 95%. The labeled albumin solution was filtered using a low protein binding 0.22 µm filter (Millex GV). Finally, 500 MBq of the labeled albumin (0.2 mg) was further diluted with NaCl 0.9% and immediately administered to the volunteer.

Data analysis
All the digitized gamma camera images were analyzed offline by the same investigator (W.O) using Siemens ICON software. Two regions of interest were drawn in each forearm representing flexor muscles and thenar muscles. Special care was taken to avoid the presence of major veins and arteries in the region of interest. Radioactivity was expressed as counts per pixel. To correct for background activity, the final result was expressed as the percentage difference between the experimental (non-dominant) arm and control arm ('targeting'):
Targeting = $(ROI_{experimental\ arm} - ROI_{control\ arm}) \times 100\% / ROI_{control\ arm}$
in which ROI represents region of interest (flexor or thenar muscle). The effect of ischemic exercise on targeting of annexin A5 was statistically analyzed with a repeated measures ANOVA with time as a within-subject factor and group as between subject factor. For this analysis, the first time point (immediately after reperfusion) was excluded because of the confounding effect of postischemic reactive hyperemia. Because this analysis revealed a significant interaction between time and group, results were also expressed as change in targeting from 1 to 4 hours after reperfusion. For each timepoint, post hoc analysis was performed with a one-way ANOVA followed by Scheffé's test for post hoc comparisons (SPSS for windows, release 10.0.7, SPSS Inc., Chicago, Il). Pilot studies have shown that forearm blood flow as measured by plethysmography increased from 5±1 to 33±3 ml/min/dl forearm (n=6, mean±SE) directly after ischemic exercice and reduced towards baseline levels within one hour.

RESULTS

Groups were similar with respect to age, weight, height, blood pressure, random plasma glucose concentration, maximal voluntary handgrip force, and duration of the ischemic exercise. All volunteers quickly and uneventfully recovered from the ischemic exercise.

Directly after injection of radiolabeled annexin A5, a large increase in activity was observed in all 3 groups in the thenar muscles of the experimental compared with the control arm (69 ± 4, 63 ± 8 and 78 ± 13% in Isch. Ex., IP+Isch. Ex., ADO+Isch. Ex. respectively; *P*=0.50 for between-group comparison, one way ANOVA). This initial increase almost completely disappeared at 1 hour after reperfusion in all groups and is interpreted as the result of postocclusive hyperemia (Table 1).

Table 1: Course in Annexin A5 targeting (% difference between experimental and control side; mean±SE). Isch. Ex.: Ischemic Exercise; IP: Ischemic Preconditioning; ADO: intra-arterial adenosine; PHENT: intra-arterial phentolamine. The overall ANOVA for repeated measurements showed a significant interaction between group and time (*P*=0.000 and *P*=0.006 for thenar and flexor muscle respectively). *: P<0.01 for effect of time (ANOVA for repeated measurements; only t=1 and t=4 included in this analysis). †: P<0.05 and ‡: P<0.01 versus Isch. Ex. (One way ANOVA, followed by Scheffé's post hoc test; PHENT+Isch.Ex. was excluded from this analysis). #:P<0.005 versus ADO+Isch.Ex. for interaction between time and group (Repeated measures ANOVA).

	Time after reperfusion (hours)			
	0	1	2	4
Thenar muscle				
Isch. Ex. (n=10)	69 ± 4	19 ± 1	23 ± 2	26 ± 3*
IP+Isch. Ex. (n=8)	63 ± 8	9 ± 3†	8 ± 2‡	6 ± 1‡
ADO+Isch.Ex. (n=9)	78 ± 13	15 ± 2	-	10 ± 3‡
PHENT+Isch.Ex. (n=10)	-	21 ± 7	-	25 ± 7*#
Flexor muscle				
Isch. Ex. (n=10)	63 ± 6	5 ± 1	7 ± 1	9 ± 2*
IP+Isch. Ex. (n=8)	63 ± 5	4 ± 1	3 ± 1	2 ± 2
ADO+Isch.Ex. (n=9)	86 ± 9	8 ± 3	-	6 ± 3
PHENT+Isch.Ex. (n=10)	-	7 ± 2	-	8 ± 2

At the end of the subsequent three hours, the annexin labeling in the ischemic hand of the Isch. Ex.-group was 26±3% greater than in the control hand (figure 1). In contrast, after ischemic preconditioning (IP+Isch. Ex.) and after previous infusion of adenosine (ADO+Isch. Ex.), this difference in activity further decreased to 6 ± 1 and 10 ± 3 % , respectively (Table 1).

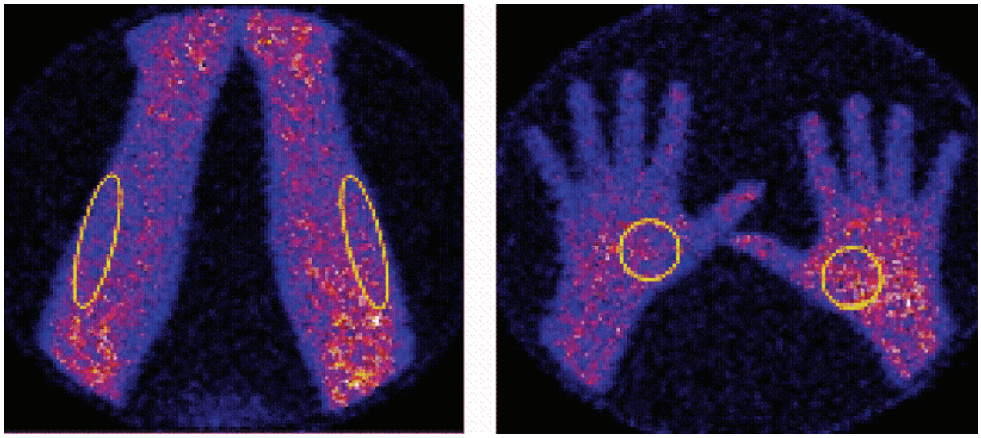

Figure 1: Typical image of flexor (left) and thenar (right) muscle at four hours of reperfusion. The regions of interest are marked. In this individual, the percentage difference in radioactivity (counts/pixel) between experimental and control side was 37% and 10% for thenar and flexor muscle respectively. In this particular experiment, ischemic exercise was performed without previous preconditioning. Exercise is required during ischemia for targeting of annexin A5. In addition, the distribution of radioactivity is in accordance with uptake in muscle instead of bone or skin.

Consequently, the increase over time of annexin A5 targeting from 1 to 4 hours after reperfusion was significantly blunted in the IP+Isch.Ex. and in the ADO+Isch.Ex.-group compared with the Isch.Ex.-group (Fig. 2).

To assess the effect of vasodilation *per se*, adenosine was replaced by phentolamine (PHENT) in an extra series of experiments. In this PHENT+Isch.Ex.-group, annexin A5 targeting increased over time, resulting in a difference between the two hands of 25 ± 7 %, a number closely resembling the results of the Isch.Ex.-group. As a consequence, annexin A5 targeting in the PHENT+Isch.Ex.-group was significantly higher compared with the IP+Isch.Ex.- and the ADO-Isch.Ex.-group (table 1, fig. 2).
In contrast to annexin A5, Tc-99m-albumin was not retained in the experimental hand after ischemic exercise. The difference in activity between the hands was 2 ± 0.5 (range: 1 to 3) % and 0 ± 1 (-3 to 1) % at one and four hours of reperfusion, respectively (n=3) and significantly differed from the Isch.Ex.-group in which Tc-99m-annexin A5 was used instead of albumin (mean ± SE; $p<0.01$ for effect of group; $p<0.05$ for interaction between group and time; repeated measures ANOVA).

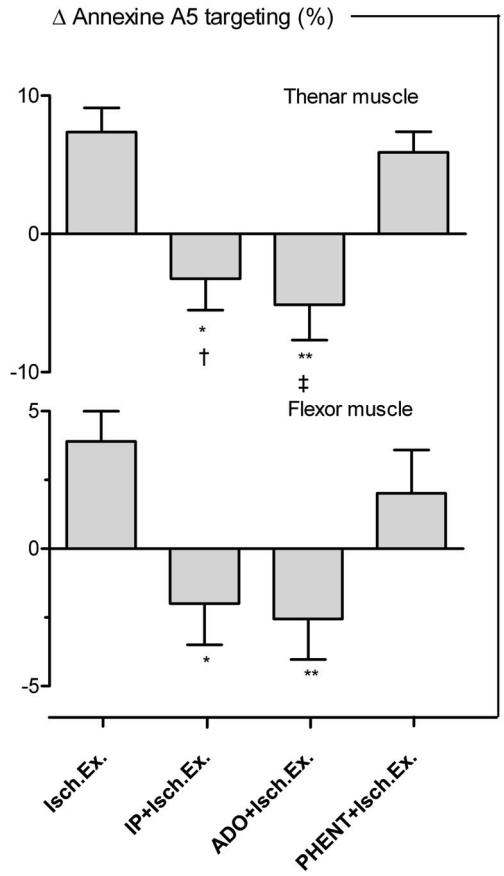

Figure 2: Change in percentage difference between experimental and control side from 1 to 4 hour after reperfusion. Isch. Ex.: Ischemic Exercise; IP: Ischemic Preconditioning; ADO: adenosine; PHENT: phentolamine*: P<0.05 versus Isch. Ex.; **: P<0.01 versus Isch. Ex.; †: P<0.05 versus PHENT+Isch.Ex.; ‡: P<0.01 versus PHENT+Isch.Ex.

In a final control experiment, Tc-99m-annexin A5 was not injected directly after reperfusion, but one hour later. With this approach, annexin A5 targeting to the thenar muscle was only 7 ± 2 (range: 5-11) % at four hours after reperfusion (n=3), which was significantly different from the 26 ± 3 % (see table 1, Isch.Ex.-group) when annexin A5 was injected immediately upon reperfusion (P<0.01 for effect of group; repeated measures ANOVA).

In general, targeting of radiolabeled annexin A5 showed a similar pattern for the flexor muscle, although less pronounced than for the thenar muscle (Table 1 and Fig. 2). Results did not relevantly change when the statistical analysis was refined with workload as a covariate.

DISCUSSION

This study reveals three novel findings: (1) ischemic handgrip increases uptake of annexin A5 in the hand and forearm; (2) this targeting of annexin A5 is prevented by a previous bout of 10 minutes ischemia and 10 minutes reperfusion (ischemic preconditioning); and (3) intra-arterial infusion of adenosine provides protection against ischemia-reperfusion (pharmacological preconditioning). These observations were most pronounced in the thenar muscle of the hand but also occurred in the flexor muscle and resemble the effect of ischemic preconditioning and intra-arterial infusion of adenosine on infarct size in skeletal muscle of pigs after a more prolonged ischemic insult (432, 541). Thus, our observations are in accordance with our hypothesis that annexin A5 scintigraphy validly detects ischemia-reperfusion injury, ischemic preconditioning and pharmacological modulation of ischemia-reperfusion injury.

We did not observe retention of radiolabeled albumin in the experimental arm, indicating that the observed targeting of annexin A5 is not caused by non-specific targeting such as changes in vascular permeability or reactive hyperemia. Delay of the annexin A5 injection by 1 hour did not result in uptake of annexin A5. Finally, in a pilot study in 6 healthy volunteers the arteriovenous difference in plasma creatine kinase (CK) activity across the experimental forearm did not change in response to this ischemic exercise, excluding relevant skeletal muscle necrosis (data not shown). Taken together, these observations are fully compatible with a short-lasting availability of annexin A5 binding sites after this relatively mild ischemic stimulus and may provide an argument against the occurence of apoptosis in this model. This finding contrasts with the long-lasting availability of annexin A5 binding sites after myocardial infarction or apoptosis as observed by others (136, 137, 544, 546). Our observation is in agreement, however, with reversible availability of externalized phosphatidylserines in response to mild hypoxia or ischemia as has been reported by others (135, 548, 549).

Alternatively, one might argue that targeting of annexin A5 in our experiments is mainly driven by reactive hyperemia. Although this concept provides an explanation for the lack of uptake of annexin A5 when it is injected 1 hour after reperfusion, it does not explain why annexin A5 targeting was prevented by ischemic preconditioning or adenosine infusion. Reactive hyperemia was similar for the 3 groups, as reflected by a similar distribution of annexin A5 directly after reperfusion: nevertheless significant differences in retention of annexin A5 were observed 4 hours after reperfusion. Our interpretation that reactive hyperemia is not significantly involved in targeting of annexin A5 after ischemic exercise is further supported by the lack of uptake of Tc-99m-albumine. The observed action of ischemic- and pharmacological preconditioning on annexin A5 targeting after ischemic exercise closely resembles the effect of these interventions on infarct size as demonstrated in various animals and organs including skeletal muscle (432, 541, 550), and a clinical trial in the heart (551). This further indicates that this technique detects relevant early signs of ischemic exercise and reperfusion-induced injury and can be used for future pharmacological research to evaluate agents intended to protect against ischemia-reperfusion injury in humans *in vivo*.

In this study, we used phentolamine as a control vasodilator for adenosine. In contrast to other vasodilators such as NO-donors or calcium channel blockers, phentolamine does not have a known direct effect on cellular tolerance against ischemia-reperfusion. It should be emphasized, however, that the vasodilator action of phentolamine is limited given a certain baseline α-adrenergic tone.

We did not measure the vasodilator response to adenosine and phentolamine in these experiments. It is well known from previous studies with these drugs that forearm blood flow measured with strain-gauge plethysmography increases with a factor of 3 to 5 in response to phentolamine (552, 553) and a factor of 10 in response to adenosine (554). Therefore, our observation with phentolamine indicates that an increase in flow prior to ischemic exercise to approximately 10 ml/min/dl forearm tissue does not affect annexin A5 scans after ischemic exercise. However, our observation does not exclude that part of the protective action of adenosine could involve vasodilation, for example by increasing shear stress and subsequent release of nitric oxide before the ischemic exercise. Therefore, the mechanism of protection of exogenous adenosine may be different from ischemic preconditioning. However, this possibility also occurs in the animal studies, exploring the protective action of adenosine in the heart or skeletal muscle with infarct size as an end point. The present study was not intended to elucidate the mechanism of adenosine-induced protection.

Models previously used to study ischemic preconditioning in humans have important pitfalls. Epidemiological studies have shown that preinfarct angina reduces infarct size (395, 398, 555). However, these studies may have been biased by differences in reperfusion time, which is significantly shorter in the patients with preinfarct angina (556). Repeated PTCA reduces lactate formation and ischemia-associated ECG changes. Like infarct size in animal models, these surrogates for ischemia-reperfusion injury are responsive to adenosine receptor antagonists and glibenclamide (407, 439). However, possible recruitment of collateral circulation, which could be reduced by the pharmacological treatment, complicates interpretation of these observations. Changes in venous lactate do not necessarily reflect differences in ischemic injury but may result from differences in ischemic load. Observations in animals indicate that electrocardiography is a poor surrogate end point to detect ischemia-reperfusion injury, especially when pharmacological interventions such as glibenclamide interfere with the electrophysiological properties of the sarcolemma (413). Finally, repeated PTCA is a complicated procedure with inherent risk which makes this model difficult to use for pharmacological screening in humans *in vivo*. An elegant model of ischemic preconditioning is restricted to patients undergoing coronary artery bypass surgery (125). This limitation hinders efficient screening of pharmacological or physiological interventions for their potential to interfere with ischemic preconditioning in conscious persons. In contrast to the limitations of these methods, ischemic forearm exercise followed by annexin A5 scintigraphy is not biased by collateral circulation, detects membrane changes that directly result from ischemia-reperfusion injury, and can easily be applied to volunteers with minimal risk of serious complications.

In conclusion, annexin A5 scintigraphy reliably detects ischemia-reperfusion injury and a protective effect of interventions that are known to reduce infarct size in skeletal muscle. Our observations support the use of this model as a possible screen for pharmacological interventions that aim at reducing ischemia-reperfusion injury in clinically relevant organs such as the heart or brain. For this purpose, however, further research, including elucidation of signal transduction pathways, is needed to validate extrapolation of findings in the forearm to other organs.

ACKNOWLEDGEMENTS

The contribution of Dr. G.A. Rongen has been made possible by a fellowship from the Royal Netherlands Academy of Arts and Sciences. N. P. Riksen is an MD-clinical research trainee financially supported by the Netherlands Organization for Scientific Research (ZonMw).

Additional financial support was obtained from the European Union (project number: QLK1-CT-2000-00069). The NAS 2020 kits for preparation of Tc-99m-Hynic-rh-Annexin A5 were donated by Theseus Imaging Corparation, Boston, MA, USA. The aurthors want to thank Dr. W. van Heerde (Department of Hematology, UMC Nijmegen St Radboud) for his critical and helpful comments and discussions, N. Jacobs (Department of Hematology, UMC Nijmegen St Radboud) for her help with the analysis of plasma creatine kinase and I. Drost (Department of Pharmacology-Toxicology, UMC Nijmegen St Radboud) for her technical assistance in the experiments with phentolamine and Tc-99m-albumin.

CHAPTER 4.4

ORAL THERAPY WITH DIPYRIDAMOLE LIMITS ISCHEMIA-REPERFUSION INJURY IN HUMANS

Clin Pharmacol Ther 2005;78:52-9

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ABSTRACT

Background: Adenosine receptor stimulation induces several effects that could limit ischemia-reperfusion injury. We hypothesize that treatment with the nucleoside uptake inhibitor dipyridamole increases endogenous adenosine and limits ischemia-reperfusion injury in humans.

Methods: Ischemia-reperfusion injury was studied in forearm skeletal muscle by ^{99m}Tc-annexin A5 scintigraphy. Ischemia-reperfusion injury was induced by unilateral forearm ischemic exercise. Immediately upon reperfusion, ^{99m}Tc-annexin A5 was administered intravenously and ischemia-reperfusion injury was expressed as the percentage difference in radioactivity between the experimental arm and the control arm 1 and 4 hours after reperfusion. Targeting was quantified in the region of the thenar muscle and forearm flexor muscles. This approach was used in 9 healthy male volunteers, after a one-week treatment with dipyridamole (200 mg slow-release, twice daily) and in 23 control subjects.

Results: Dipyridamole treatment significantly reduced annexin A5 targeting in skeletal muscle compared with the control group (thenar region: 13 ± 7 % versus 22 ± 15 % at 1 hour after reperfusion and 9 ± 6 % versus 27 ± 13 % at 4 hours for dipyridamole and control groups, respectively, P=0.01; flexor region: 4 ± 8 % versus 7 ± 6 % at 1 hour after reperfusion and 1 ± 4 % versus 10 ± 9 % at 4 hours for dipyridamole and control groups, respectively, P=0.01).

Conclusions: One week of oral treatment with the nucleoside uptake inhibitor dipyridamole (200 mg, slow-release, twice daily) significantly limits ischemia-reperfusion injury in humans *in vivo*, as assessed by ^{99m}Tc-annexin A5 scintigraphy of forearm skeletal muscle.

INTRODUCTION

Despite state-of-the-art reperfusion therapy, the 30-day mortality rate of acute myocardial infarction is still approximately 7 % (386). In addition, as a result of improved survival after acute myocardial infarction, the incidence and prevalence of chronic heart failure, which is often caused by ischemic death of cardiomyocytes, increase rapidly. Therefore the development of new strategies to reduce morbidity and mortality rates once infarction has occurred could provide substantial clinical benefit.

The most potent endogenous protective mechanism against ischemia-reperfusion injury of the myocardium, other than early reperfusion, is ischemic preconditioning. Initially described by Murry *et al*, this phenomenon describes the powerful infarct size-limiting effect of brief sublethal periods of ischemia and reperfusion preceding a sustained period of ischemia (122). The endogenous purine nucleoside adenosine, which is released during the preconditioning ischemia, has been shown to play a pivotal role in the protection afforded by preconditioning (86). Endogenous adenosine also has several additional effects other than inducing preconditioning, which ultimately protect against ischemia-reperfusion injury, such as vasodilation, inhibition of inflammation, and inhibition of platelet aggregation and the formation of thromboemboli (8, 90, 92).

Because of these beneficial effects, pharmacological elevation of the concentration of endogenous adenosine is an attractive target to attenuate ischemia-reperfusion injury. The nucleoside uptake inhibitor dipyridamole effectively increases the plasma concentration of adenosine and may, therefore, serve this goal (111). Because myocardial ischemic events are seldom planned or accurately predicted, it is desirable to offer a sustained protection against ischemia and reperfusion in patients who are at risk for myocardial ischemia. In the present study we, therefore, aimed to determine whether oral treatment with dipyridamole limits ischemia-reperfusion injury in humans *in vivo*. For this purpose, we used the recently developed and well-validated technique of ^{99m}Tc-annexin A5 scintigraphy to detect ischemia-reperfusion injury in forearm skeletal muscle in healthy male volunteers (140). This model is based on the specific and high-affinity binding of annexin A5 to phosphatidylserine residues. Loss of membrane asymmetry occurs shortly after an ischemic insult and results in phosphatidylserine exposure on the outer membrane leaflet, either as early sign of apoptosis or as reversible event, preceding commitment to apoptotic cell death (134, 135). By labeling recombinant annexin A5, it is possible to visualize this loss of membrane asymmetry *in vivo*. Using the protocol described in the present study, we previously showed that ischemic exercise of forearm muscle increases targeting of annexin A5 and that this can be prevented by ischemic preconditioning as well as by the infusion of adenosine into the brachial artery (140).

METHODS

Subjects

After approval of the protocol by the Institutional Review Board of the Radboud University Nijmegen Medical Center, 32 healthy male volunteers signed written informed consent statements before participation in the study. They had no history of cardiovascular disease or asthma and did not use any medication. In all participants a physical examination, electrocardiography and laboratory investigation were performed to exclude cardiovascular and pulmonary disease, hypertension and diabetes mellitus. Because caffeine is a potent adenosine receptor antagonist, all

volunteers were asked to abstain from caffeine-containing beverages for at least 24 hours before each visit on days 1, 6, and 7 of the study.

Experimental protocol

All participants in the active treatment group (n=9) were treated orally for one week with 200 mg slow-release dipyridamole twice daily in an open-label design (Persantin Retard® 200 mg, Boehringer-Ingelheim, Alkmaar, the Netherlands). On days 1, 6, and 7 of the study they visited our department in the morning for hemodynamic monitoring, venous blood sampling and supervised administration of the study medication. Heart rate and blood pressure were measured with an automated blood pressure device (Dinamap™, Critikon, Inc., Tampa, U.S.A.) on the dominant arm, with 4 measurements performed at 3-minute intervals, starting after 5 minutes of supine rest. On day 1, blood pressure and heart rate were measured before administration of the first dose and at 2 hours thereafter. On day 6, hemodynamic measurements were performed and blood was drawn for determination of plasma dipyridamole concentration before administration of the morning dose. On day 7, heart rate and blood pressure were measured again, before and 2 hours after administration of the morning dose of dipyridamole. Venous blood samples were taken on these two occasions for determination of the plasma concentration of dipyridamole and caffeine. On day 7, immediately after the final hemodynamic measurement (approximately 145 minutes after administration of the final dose of dipyridamole), the ischemic-exercise experiment was performed as previously described (see next paragraph) (140). This active treatment group was compared with a control group of 23 healthy male volunteers who only performed the ischemic-exercise experiment without pretreatment. A part of this control group (n=10) has been reported on previously in a recent study from our group (140).

Ischemic-exercise protocol

In brief, all volunteers were studied in a sitting position after cannulation of an antecubital vein of the dominant forearm. Maximal voluntary contraction was determined in the non-dominant arm with a handgrip dynamometer (Baseline Hydrolic Hand Dynamometer, Fabrication Enterprise Inc., Irvington, New York, USA). Subsequently, the circulation of the non-dominant arm was occluded for 10 minutes by inflation of an upper arm cuff to 200 mmHg. Simultaneously, the subjects performed rhythmic isometric handgripping at 50% of maximal voluntary contraction for 5 seconds every 10-second period until exhaustion. The total duration of ischemia was 10 minutes. Immediately upon reperfusion, 0.1 mg of hydrazinonicotinamide(HYNIC)-derivatized recombinant human annexin A5 (NAS2020, Theseus Imaging Corporation, Boston MA), radiolabeled with 450 MBq Tc-99m, was administered intravenously into the dominant arm. Both forearms and hands were imaged at 1 and 4 hours after injection by use of a gamma camera (Siemens Orbiter, Hoffman Estates, Illinois, USA, equipped with low-energy high resolution collimators) connected to a Hermes Gold image processing system (Nuclear Diagnostics, Stockholm, Sweden) as previously described (140).

Radiolabeled annexin A5 was freshly prepared before each experiment as previously described (8).

***Ex vivo* determination of nucleoside uptake inhibition**

In the last three participants of the active treatment group, blood was drawn in EDTA-containing Vacutainer blood collecting tubes on day 1, before administration of the first dipyridamole dose, and on day 7, immediately before the start of the experiment, approximately 145 minutes after administration of the final dose for *ex vivo* determination of transport inhibition of adenosine and uridine. Immediately after the venous sample was taken, the blood was centrifuged, the erythrocytes were washed twice in normal saline solution and resuspended in MOPS-buffer to obtain a 20% solution.

For uridine transport measurements, a 50-µl uridine solution was added to 100 µl 10% erythrocytes in MOPS buffer to obtain a final concentration of 10, 30, 100, 200, 400, and 1000 µM. After 3 seconds, uridine uptake was completely blocked by 100 µl 25 µM dipyridamole, and the erythrocytes were isolated by immediate centrifugation through a dibutylphthalate layer. After removal and washing of the upper layer, the erythrocytes were lysed with Triton X-100 and treated with perchloric acid for protein precipitation. After centrifugation, the uridine concentration in the supernatant was determined by HPLC.

For adenosine uptake determinations, adenosine (in a final concentration of 3 µM) was added to 100 µl of 1% erythrocytes in Tris-NaCl-buffer. After 0, 3, 6, 10, and 15 minutes, adenosine uptake and deamination were completely blocked by high dose dipyridamole (10 µM) and erythro-9-(2-hydroxynon-3-yl)-adenine (8 µM), respectively. Subsequently, the cells were separated from the supernatant by centrifugation through a dibutylphthalate layer, and the adenosine concentration in the supernatant was determined as described later.

Analytical procedures

Plasma caffeine concentrations were determined by use of reversed-phase HPLC with UV detection set at 273 nm according to Schreiber-Deturmeny and Bruguerolle (147). Plasma dipyridamole concentrations were determined in deproteinised plasma by use of HPLC with fluorescence detection set at 286/470 nm as previously described by Wolfram and Bjornsson (557).

In the *ex vivo* nucleoside uptake experiments, uridine concentration was determined by HPLC with UV-detection set at 254 nm using a Polaris C18 column. For the mobile phase, 10 mM tetrabutylammoniumhydrogensulphate (TBAHS) in 0.1% acetic acid was used. Uridine uptake was expressed as nmol/min/mg protein in the membranous fraction, determined according to the well-documented Lowry-assay. The adenosine concentration was determined with reversed-phase HPLC with UV detection set at 260 nm. Adenosine was separated by a linear gradient of 2% acetonitril (in 10 mM TBAHS, 20 mM $NH_4H_2PO_4$, pH 6.0) to 35% acetonitril in 15 minutes at 1 ml/min.

Statistical analysis

All data are presented as mean ± SD unless otherwise stated. Because not all baseline characteristics showed a Gaussian distribution, the Mann Whitney U-test was used to compare groups. Effects of dipyridamole on hemodynamic parameters were tested with a paired-sample 2-sided t-test.

All of the digitized gamma camera images were analyzed off-line by the same investigator (W.O) by use of Hermes Gold software. Two predefined regions of interest were drawn in each forearm representing flexor muscles and thenar muscles respectively. Annexin A5 targeting was expressed

as the percentage difference between the experimental (nondominant) arm and the control arm ('targeting') as previously described (8). The differences between the groups and the interaction between group and time were analyzed with an ANOVA for repeated measures (SPSS for windows, release 10.0.7, SPSS Inc., Chicago, Il). In this model we corrected for workload, which was defined as the product of maximal voluntary force and duration of ischemic exercise (ANOVA for repeated measures with workload as covariate).
All *ex vivo* measurements were performed in duplicate and averaged for each subject. The *ex vivo* uridine uptake was plotted for each subject according to Michaelis-Menten kinetics. The adenosine uptake was plotted according to one-phase exponential decay and half lives were calculated for each subject. The calculated V_{max} and half lives were compared with paired t-tests.

RESULTS

Subjects

The demographic characteristics of both groups are summarized in table 1. Baseline heart rate was higher in the dipyridamole-treated group (P=0.05). Other baseline parameters were not significantly different between groups (P>0.1). The plasma caffeine concentration in the dipyridamole group was <0.07 mg/l in 8 subjects, but 1.19 mg/l in one subject, indicating that the instructions regarding caffeine intake were not adequately followed by the latter subject. However, exclusion of this subject did not change the overall results. Trough plasma dipyridamole concentrations in the morning on day 6 and 7 were 0.79 ± 0.41 mg/l and 0.70 ± 0.46 mg/l respectively, indicating steady state. Peak plasma concentration on day 7, immediately before the experiment, averaged 1.33 ± 0.33 mg/l. Both peak and trough levels are comparable to the plasma concentrations of dipyridamole as mentioned in the large European Stroke Prevention Study 2 (ESPS-2) (558).

Table 1: Baseline characteristics of the study groups; *Part of this control group has been published previously by our group (140). #Blood pressure measured by auscultation with sphygmomanometry while the subjects were in the supine position, and heart rate was measured by pulse frequency counting. †P=0.05 versus control group.

	Control group*	Dipyridamole group
Number	23	9
Age (years)	23 ± 3	25 ± 5
Height (cm)	181 ±7	182 ± 7
Weight (kg)	75 ± 9	75 ± 11
Body mass index (kg/m^2)	23 ± 2	23 ± 2
Systolic blood pressure (mmHg)#	131 ± 7	130 ± 8
Diastolic blood pressure (mmHg)#	74 ± 9	76 ± 6
Heart rate (bpm)#	69 ± 10	77 ± 10†
Random glucose (mmol/l)	4.9 ± 0.7	5.0 ± 0.8
Workload (kg×s)	9803 ± 3528	9958 ± 1727

Hemodynamic parameters are depicted in figure 1. The first dose of dipyridamole did not change systolic blood pressure but did significantly decrease diastolic blood pressure from 65 ± 3 to 60 ± 6 mmHg (P=0.005) and increased heart rate from 66 ± 10 to 74 ± 8 beats/min (P=0.003). This hemodynamic effect is consistent with earlier reports on hemodynamic changes in response to intravenously administered dipyridamole (109). On day 7, ingestion of the dipyridamole dose did no longer induce any immediate hemodynamic effects. Moreover, heart rate and blood pressure immediately before the main experiment on day 7 did not differ from baseline values obtained on day 1, although the diastolic blood pressure tended to be lower on day 7 (62 ± 5 versus 65 ± 3 mmHg, P=0.06).

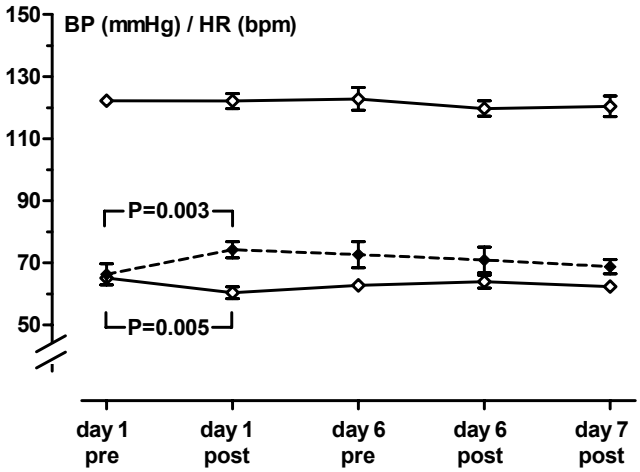

Figure 1: Systolic and diastolic blood pressure (open symbols) and heart rate (filled symbols, dotted line) on day 1, 6 and 7 of the study before (pre) and 2 hours after (post) administration of the morning dipyridamole dose (mean ± SE). On day 1, heart rate was increased (P=0.003) and diastolic blood pressure decreased (P=0.005) after administration of the first dose of dipyridamole.

Annexin A5 scintigraphy

Annexin targeting at 1 and 4 hours after reperfusion is illustrated in figure 2.

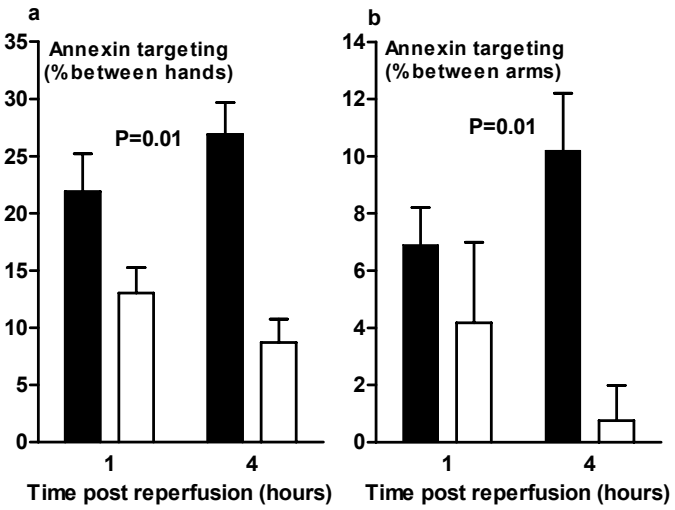

Figure 2: Annexin targeting in the thenar region of the hand (a) and in the flexor region of the arm (b) at 1 and 4 hours after reperfusion (mean ± SE). Open bars represent the dipyridamole group, the filled bars represent the control group. Annexin targeting was reduced in the dipyridamole-treated group, in both the flexor region and the thenar region (P=0.01 for interaction between group and time, analysis of variance for repeated measures).

In the thenar region of the hand, the targeting of annexin A5 was significantly reduced in the dipyridamole group compared with the control group (P=0.01 for between-group effect, P=0.01 for interaction between group and time). Annexin A5 targeting was also reduced by dipyridamole in the flexor region (P=0.04 for between-group effect, P=0.01 for the interaction between group and time).

***Ex vivo* determination of nucleoside uptake inhibition**

Uridine uptake into the erythrocytes via the dipyridamole-sensitive nucleoside transporter was significantly inhibited on day 7 compared with day 1 (before the first dose of dipyridamole): V_{max} decreased from 328 ± 28 nmol/min/mg protein to 169 ± 28 nmol/min/mg, n=3, P=0.02, figure 3a. In addition, the uptake of adenosine was significantly inhibited on day 7 compared with day 1 (half life increased from 8.0 ± 0.7 minutes to 9.9 ± 0.8 minutes, n=3, P=0.004, figure 3b).

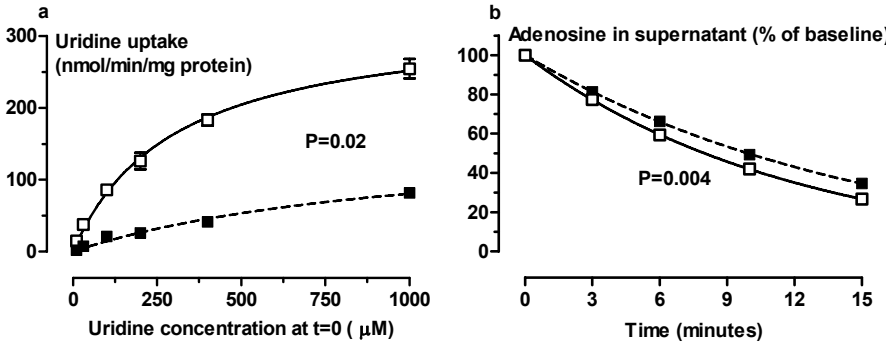

Figure 3: *Ex vivo* uridine (a) and adenosine (b) uptake of isolated erythrocytes (mean ± SE). Uridine uptake is depicted, expressed as nmol/min/mg protein (a). V_{max} was lower after dipyridamole treatment (*P*=0.02). Figure b shows the concentration of adenosine in the supernatant 3, 6, 10 and 15 minutes after the addition of adenosine to the erythrocyte suspensions. The erythrocytes were obtained on day 1, before administration of the first dose of dipyridamole (open squares, n=3), and on day 7 two hours after administration of the last dose of dipyridamole (filled squares, n=3). Half life was increased after dipyridamole treatment (*P*=0.004).

DISCUSSION

The main finding of this study is that a one-week treatment with the nucleoside uptake inhibitor dipyridamole, with a dose of 200 mg twice daily, effectively inhibits cellular adenosine uptake and potently limits ischemia-reperfusion injury of forearm skeletal muscle in humans *in vivo*. Extrapolating this finding, dipyridamole may well confer additional clinical benefit in patients who are at risk for myocardial ischemia.

Originally introduced as an antianginal drug in 1959, dipyridamole has since been used especially as an antithrombotic drug, because of its inhibitory effect on platelet aggregation (103, 559). Only in the last decade has dipyridamole been used in experimental settings to modulate ischemia-reperfusion injury. Results in animal studies have been equivocal. Although short-term pretreatment with dipyridamole protected against myocardial stunning in the rabbit heart and potentiated the infarct size-limiting effects of ischemic preconditioning in rabbit and canine hearts (64, 191, 560), short-term pretreatment with dipyridamole without preconditioning did not reduce infarct size in rabbit and canine hearts (64, 191, 542). In humans, several experimental studies suggested clinical beneficial effects of dipyridamole on ischemia-reperfusion injury, but the methods used in these studies all suffer from methodological shortcomings (436, 455, 561-565). In patients with coronary artery disease it was shown that short-term intravenous administration of dipyridamole, as well as four-day oral treatment with dipyridamole, increases tolerance to dipyridamole stress-echocardiography (561, 564). However, pretreatment with dipyridamole might well have influenced sensitivity to dipyridamole, thus affecting ischemic load and not resistance to ischemia. In patients with stable coronary artery disease, short-term intravenous pretreatment with dipyridamole, but not chronic oral treatment with dipyridamole, increased tolerance to exercise testing (562, 565). Increased collateral circulation could have confounded these findings. Finally,

increased collateral circulation might also have affected the finding that intracoronary administration of dipyridamole prior to PTCA in patients with symptoms of coronary artery disease increased tolerance to balloon inflation (436, 455, 563).
With ischemic or pharmacological preconditioning, the time frame of protection is limited. Because sustained pharmacological protection is preferred in patients at risk for myocardial ischemia, several studies have explored whether the short-term protection afforded by pharmacological preconditioning with exogenous or endogenous adenosine persists in time. Although the rabbit heart showed desensitization to the protective effect of a selective adenosine A_1-receptor agonist, when administered as 72-hour continuous infusion (508), protection was maintained after repeated intravenous bolus infusions at 48-hour intervals for 10 days (509). Moreover, chronic oral administration of dipyridamole for 2-6 weeks in guinea pigs provided sustained protection to stunning and creatine kinase release (510). In the current study we showed that oral therapy with dipyridamole for one week also provides sustained protection against ischemia-reperfusion in humans *in vivo*.

Although this study is an experimental study with surrogate end-points, on the basis of our results, one would expect to also see benefits from dipyridamole therapy in clinical trials. Protection by dipyridamole in patients at risk for myocardial ischemia would lead to attenuation of infarct size and, subsequently, less progression to heart failure and a reduction in mortality. However, the vast majority of end-point studies are characterized by disappointing results (456). There are several possible explanations for this discrepancy, although these are speculative. Increased tolerance to ischemia will not decrease the incidence of vascular events but rather will limit infarct size and, subsequently, the incidence of heart failure and death once infarction has occurred. However, most studies use the incidence of vascular events as primary end-point and when vascular death was reported, studies were often underpowered for this end-point (456). Moreover, when vascular death was one of the endpoints, most studies were concentrated on patients with cerebrovascular disease and not coronary artery disease. As timely reperfusion still remains indispensable for protection to occur, and reperfusion in cerebral ischemic events occurs less often than in cardiac events, the protection afforded in these patients might be expected to be less than in patients who are prone to coronary events.

The observed effects of dipyridamole are most likely mediated by an increase in the endogenous adenosine concentration. Although dipyridamole has several other effects besides nucleoside uptake inhibition, previous reports showed that the protective effect of dipyridamole was completely prevented by the adenosine receptor antagonist theophylline, as were the hemodynamic effects of dipyridamole in humans (109, 191). Only a few studies have directly shown that oral treatment with dipyridamole indeed increases the endogenous adenosine concentration, because measurement of adenosine is severely complicated by its extremely short half life (51, 111). In the present study, we convincingly showed that dipyridamole, in the dose given, effectively inhibits uptake of both uridine and adenosine in erythrocytes. Because erythrocytes lack uridine kinases and phosphorylases, this nucleoside can be very useful to reliably obtain the transport characteristics of the equilibrative nucleoside transporter (566). On the contrary, adenosine is rapidly deaminated and rephosphorylated after uptake into the cell. In the

normal situation, intracellular deamination of adenosine, rather than its transport into the cell, is the rate-limiting step in the overall catabolism of adenosine (567). Consequently, at least 90% occupancy of the transporter by a nucleoside uptake inhibitor is required to inhibit adenosine breakdown *ex vivo*, which explains why, in our study, the disappearance of uridine is more potently inhibited than adenosine (567).

In this study the healthy volunteers were treated with extended-release preparations of dipyridamole in a dose of 200 mg twice daily, which is the recommended dose in the Netherlands for the secondary prevention of cerebrovascular events. The safety of this dose has been evaluated extensively in the ESPS-2 study (558). Short-term administration of dipyridamole increases heart rate and decreases diastolic blood pressure (109), as also observed after the first dose of dipyridamole in our study. A long-term increase in heart rate however, is undesirable in patients at risk for cardiac ischemia. In the present study the described hemodynamic effects of dipyridamole waned after one week. Moreover, on day 7 of the study dipyridamole no longer induced any short-term effects on heart rate and blood pressure. This adaptation may have been induced by adenosine receptor downregulation. However, any receptor downregulation did not abolish the protection of dipyridamole against ischemia-reperfusion.

Several limitations of the present study need to be addressed. First, the study was open-labeled and without randomization. However, both groups were similar in baseline characteristics, except for an increased baseline heart rate in the dipyridamole group and the experimental and analytical procedures were highly standardized, leaving little room for significant confounding. Second, ischemia-reperfusion injury was assessed in the forearm skeletal muscle and not directly in the myocardium. Although extrapolating these findings to the heart must be done with great caution, the mechanisms of ischemic preconditioning in skeletal muscle and myocardium do share many common pathways (433, 434, 568). Moreover, with the use of this model, it is possible to circumvent potential confounding by collateral circulation. Finally, we studied young healthy volunteers, whereas older patients who are at increased risk for ischemia might be less amenable to pharmacological preconditioning (274).

In conclusion, the present study showed that oral treatment with the nucleoside uptake inhibitor dipyridamole, in a dose of 200 mg twice daily, potently protects against ischemia-reperfusion injury in forearm skeletal muscle of healthy volunteers, as detected with ^{99m}Tc-annexin A5 scintigraphy.

ACKNOWLEDGEMENTS

N.P. Riksen is a MD-clinical research trainee financially supported by the Netherlands Organization for Scientific Research (ZonMw). The contribution of G.A. Rongen has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. P.H.H. van den Broek was financially supported by the European Union (project number: QLK1-CT-2000-00069). We thank Dr. N. Steinmetz and Theseus Imaging Corparation (Boston, MA, USA) for kindly donating the NAS 2020 kits for preparation of Tc-99m-Hynic-rh-Annexin A5. We thank Dr. S. Levison-Keating, department of hematology, Radboud University Nijmegen Medical Center for correction of the English.

CHAPTER 4.5

CAFFEINE PREVENTS PROTECTION IN TWO HUMAN MODELS OF ISCHEMIC PRECONDITIONING

J Am Coll Cardiol 2006;48:700-7

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ABSTRACT

Objectives: We studied whether caffeine impairs protection by ischemic preconditioning (IP) in humans.

Background: IP is critically dependent on adenosine receptor stimulation. We hypothesize that the adenosine receptor antagonist caffeine blocks the protective effect of IP.

Methods: *In vivo* ischemia-reperfusion injury was assessed in the thenar muscle by ^{99m}Tc-annexin A5 scintigraphy. Forty-two healthy volunteers performed forearm ischemic exercise. In 24 subjects, this was preceded by a stimulus for IP. In a randomized double-blinded design, the subjects received caffeine (4 mg/kg) or saline intravenously prior to the experiment. At reperfusion, ^{99m}Tc-annexin A5 was administered intravenously. Targeting of annexin was quantified by region-of-interest analysis, and expressed as percentage difference between experimental and contralateral hand.

In vitro, we assessed recovery of contractile function of human atrial trabeculae, harvested during heart surgery, as functional endpoint of ischemia-reperfusion injury. Field-stimulated contraction was quantified at baseline and after simulated ischemia-reperfusion, in a paired approach with and without 5 minutes of IP, in the presence (n=13) or absence (n=17) of caffeine (10 mg/l).

Results: IP reduced annexin targeting in the absence of caffeine (from 13 ± 3% to 7 ± 1% at 1 hour, and from 19 ± 2% to 9 ± 3% at 4 hours after reperfusion, P=0.006), but not after caffeine administration (targeting 11 ± 2% and 16 ± 3% at 1 and 4 hours). *In vitro*, IP improved post-ischemic functional recovery in the control group, but not in the caffeine group (8±3% versus –8±5%, P=0.003).

Conclusions: Caffeine abolishes IP in two human models at a dose equivalent to the drinking of 2 to 4 cups of coffee.

Key Words: Ischemic preconditioning, caffeine, scintigraphy, atrial trabecula.

INTRODUCTION

Brief intermittent periods of ischemia and reperfusion render the myocardium more resistant to a subsequent more prolonged period of ischemia and reperfusion. This phenomenon, which is called ischemic preconditioning (IP), is the most powerful infarct size-limiting mechanism, other than early reperfusion (122, 272). In every animal species studied, IP reduced infarct size by approximately 75% (272). Also in humans, several experimental models of ischemia-reperfusion injury demonstrated protection by IP (123). Release of the endogenous nucleoside adenosine and subsequent stimulation of membrane-bound adenosine A_1 and A_3 receptors has been identified as pivotal mechanism in IP (86, 87, 391).

Caffeine is a non-selective competitive antagonist of adenosine receptors. The plasma caffeine concentration reached after regular coffee consumption is well within the concentration range that is needed to antagonize adenosine receptors (115). Based on this knowledge, we hypothesized that caffeine attenuates the cardioprotective effects of IP at concentrations that occur in daily life. A proof of this hypothesis may have important implications for patients who are at risk for cardiac ischemia.

To address this hypothesis, we used two complementary experimental models of ischemia-reperfusion injury in humans. *In vivo* ^{99m}Tc-annexin A5 scintigraphy of thenar skeletal muscle was used to study structural injury. This model is based on the highly specific binding of annexin A5 to phosphatidylserine residues, which are exposed on the outside of cellular membranes early after an ischemic insult (140). *In vitro* we assessed post-ischemic recovery of contractile function of human atrial trabeculae as functional endpoint of ischemia-reperfusion injury (390).

METHODS

Subjects

Forty-two healthy male volunteers participated in two *in vivo* studies. They were asked to abstain from caffeine-containing beverages for at least 24 hours before the experiment. Forty-four patients awaiting cardiac surgery agreed to participate in the *in vitro* study. Patients with atrial arrhythmias, right ventricular failure, or patients who were treated with theophylline, oral antiarrhythmics or sulphonylureas were excluded because these drugs may interfere with IP. Written informed consent was obtained from all participants. Both protocols were approved by the Institutional Review Board of the Radboud University Nijmegen Medical Center.

Experimental protocols

In vivo studies

Two randomized double-blinded studies were performed (figure 1). In all subjects, after cannulation of medial antecubital veins of both forearms, maximal voluntary contraction was determined in the non-dominant arm with a handgrip dynamometer (Baseline Hydrolic Hand Dynamometer, Fabrication Enterprise Inc., Irvington, New York, USA). To induce ischemia-reperfusion injury in thenar skeletal muscle, an ischemic exercise protocol (Isch Ex) was applied as previously described (140).

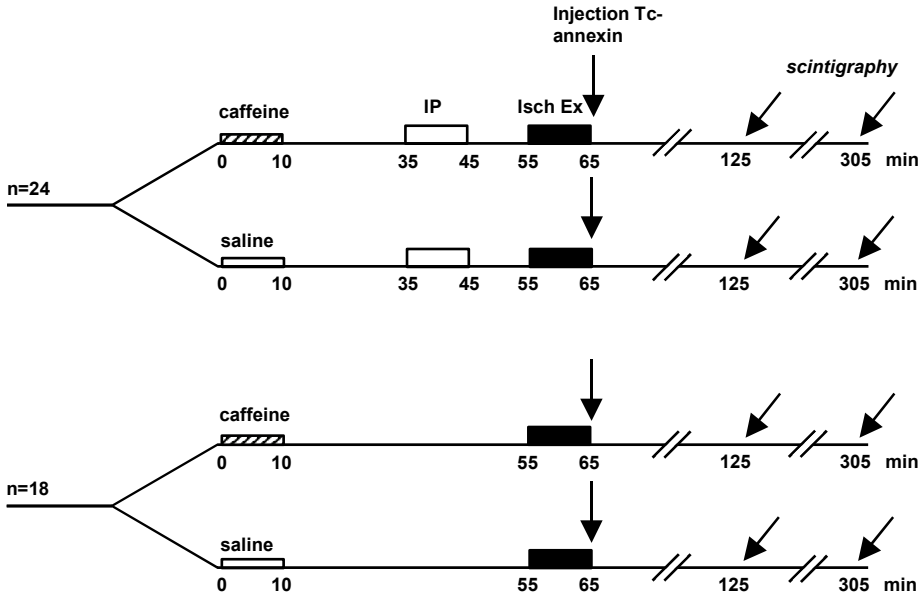

Figure 1: Schematic representation of the experimental protocol of the two randomized double-blinded *in vivo* studies. "IP" indicates the 10-minute period of ischemia (without concomitant exercise), "Isch Ex" indicates the 10-minute period of ischemia with isometric handgripping.

In short, the circulation to the forearm was occluded for 10 minutes by inflation of an upper arm cuff to 200 mmHg, which was combined with isometric contractions of the finger flexors at 50% of maximum contraction, performed rhythmically: 5 seconds of contraction followed by 5 seconds of relaxation until exhaustion. The total duration of ischemia was 10 minutes (index ischemia; Isch Ex = ischemic exercise). Immediately upon reperfusion radiolabeled annexin A5 (0.1 mg protein, 500 MBq Tc-99m) was administered intravenously into the dominant arm. Both hands were imaged simultaneously at 1 and 4 hours after injection with a gamma camera (Siemens Orbiter, Hoffman Estates, Illinois, USA, equipped with low-energy high resolution collimators) connected to a Hermes Gold image processing system (Nuclear Diagnostics, Stockholm, Sweden) as previously described (140).

In the first study (n=24), the ischemic exercise was preceded by 10 minutes of ischemia only and 10 minutes of reperfusion (IP = ischemic preconditioning). Subjects were randomized in a double-blinded fashion to receive either caffeine (4 mg/kg body weight intravenously over 10 minutes) or saline 35 minutes before the preconditioning stimulus.

To differentiate between a specific effect of caffeine on IP versus an effect on ischemia-reperfusion injury itself, a second study was performed. The set-up of this second study (n=18) was similar to the first study, but now without a preconditioning stimulus. The subjects also received caffeine or saline in a randomized and double-blind fashion.

In all subjects, blood was drawn before administration of caffeine/saline and just before the ischemic episode for the determination of plasma caffeine concentration. In the second study, we used a Fin-a-Pres tonometer (Biomedical Instrumentation, Neuilly, France) to record blood pressure and heart rate before caffeine/saline infusion and afterwards (just before the ischemic exercise episode).

***In vitro* study**

The experimental set up as described by Speechly-Dick *et al* was used with small modifications to allow simultaneous measurement of two trabeculae from one patient (390). The right atrial appendage was harvested during cardiac surgery before the introduction of the extracorporal circulation and was immediately placed in cold (4 °C) modified Tyrode's solution (NaCl 118.5 mmol/l; KCl 4.8 mmol/l; $NaHCO_3$ 13 mmol/l; KH_2PO_4 1.2 mmol/l; $MgSO_4$ 1.44 mmol/l; $CaCl_2$ 1.8 mmol/l; glucose 10.0 mmol/l and pyruvate 10.0 mmol/l), which was continuously gassed with 95% oxygen and 5% CO_2. Two atrial trabeculae (diameter<1mm; length>3mm) were dissected, vertically suspended in an organ bath and linked to a force transducer. Each trabecula was superfused with pre-oxygenated Tyrode's buffer (pO_2 500-600 mmHg). Electrical field stimulation was performed in unstretched condition at 1 Hz using platinum ring electrodes placed on both sides of the trabeculae (pulse duration 60 ms; pulse current 40 mA). After 30 minutes of stimulation at unstretched conditions to allow recovery from transportation and preparation, trabeculae were gradually stretched over 15 minutes until maximal contractile force was achieved. After 35 minutes of equilibration, a baseline recording was performed during 10 minutes. Those trabeculae that failed to produce at least 0.2 g of developed force at the end of baseline or in which the coefficient of variation of developed force exceeded 20% were excluded (n=14).

Immediately after baseline recordings, for each patient (n=30) the two trabeculae were randomly assigned to either a stimulus for IP or continued superfusion with Tyrode's solution, so that from each patient one trabecula was preconditioned and the other was not. IP was induced by 5 minutes of simulated ischemia and 5 minutes of simulated reperfusion. Simulated ischemia was accomplished by superfusing the trabeculae with substrate-free modified Tyrode's solution (7.0 mM choline chloride substituted for glucose and pyruvate) and rapid pacing at 3Hz. The superfusate was pumped into an artificial lung filled with 95%N_2 / 5%CO_2, which resulted in a low pO_2 of 10-20 mmHg.

Subsequently, both trabeculae were subjected to 90 minutes of simulated ischemia and 120 minutes of simulated reperfusion. This protocol was performed in all patients who were included (n=30). In the last 13 patients, caffeine was added to the superfusate at the end of equilibration at a final concentration of 10 mg/l.

Data recording and statistical analysis

All data are presented as mean ± SE. In the *in vivo* protocol, all digital scintigraphic images were analyzed off-line by the same blinded investigator (WJGO), using Hermes Gold software. Region-of-interest analysis was performed for the thenar muscle region in the hand. Radioactivity was expressed as counts per pixel. To correct for background activity, the final result was expressed as the percentage difference between the experimental and control hand ('targeting').

Baseline chacteristics of the four groups were analysed by one way ANOVA followed by Scheffé's test for post-hoc comparisons (SPSS for Windows, release 12.0.1).
In the two randomized studies, differences in annexin targeting between groups were analyzed with a repeated measures analysis of covariance, with time (t=1 and t=4 hours post-injection) as within-subject factor, group (with and without caffeine pretreatment) as between-subject factor, and workload (defined as the product of maximal voluntary force and duration of ischemic exercise) as covariate (SPSS for Windows, release 12.0.1).

For each contraction of the trabeculae, we calculated developed force (difference between maximal tension during contraction and minimal tension during relaxation), maximal speed of tension development during contraction (measure of systolic function) and maximal speed of tension reduction during relaxation (measure of diastolic function). These parameters were averaged for baseline, each subsequent 60-second period and the last 10 minutes of final reperfusion. Functional recovery was expressed as a percentage of baseline. Subsequently, the effect of IP was calculated as the difference in averaged percentage recovery between the two trabeculae during the final 10 minutes of reperfusion. A paired Student's t-test was used to compare control and preconditioned trabeculae, and an unpaired Student's t-test was used to compare groups with and without caffeine (SPSS for Windows, release 12.0.1).

Preparation of ^{99m}Tc-HYNIC-annexin A5

Radiolabeled annexin A5 was freshly prepared before each experiment by adding ^{99m}Tc-Pertechnetate (1500 MBq) in the presence of stannous tricine to succinimidylhydrazinonicotinamide (HYNIC)-conjugated recombinant human annexin A5 (NAS 2020, 0.275 mg per vial; Theseus Imaging Corp). For the second study (without preconditioning), recombinant human annexin A5 (obtained from Theseus Imaging Corporation) was conjugated with HYNIC in our own laboratory. The radiolabeling procedure of this HYNIC-conjugated annexin A5 was identical to that of NAS 2020.

RESULTS

Baseline characteristics

In both *in vivo* studies, subjects were randomized to caffeine or saline administration. As such, the two *in vivo* studies comprise four different groups: saline-IP-Isch Ex, caffeine-IP-Isch Ex, saline-Isch Ex, and caffeine-Isch Ex. Baseline characteristics of these four groups are shown in table 1.

There were no significant differences between the groups, except for heart rate (table 1). Atrial tissue was obtained in 44 patients. According to the predefined criteria, 14 patients were excluded from analysis. There were no significant differences in clinical characteristics between the groups with and without caffeine (table 2).

Table 1: Baseline characteristics of the groups in the *in vivo* studies.*P<0.05 versus Caffeine-Isch Ex; one way ANOVA followed by Scheffé's post-hoc test.

	Saline-IP-Isch Ex	Caffeine-IP-Isch Ex	Saline-Isch Ex	Caffeine-Isch Ex
Number	12	12	9	9
Age (years)	25±1	24±1	21±1	23±2
Weight (kg)	76±3	76±3	74±3	74±3
Height (cm)	186±2	182±2	184±3	183±2
SBP (mmHg)	124±3	129±3	126±3	130±3
DBP (mmHg)	76±2	76±3	75±2	71±3
Heart rate (bpm)	67±3	71±2*	61±2	60±3
Random glucose (mmol/l)	4.8±0.2	4.4±0.3		
Coffee intake (cups/week)	11±4	11±3	13±2	9±3
Workload (kg·s)	10000±1058	9147±594	7219±642	7179±886
Baseline plasma caffeine (mg/l)	0.3±0.1	0.4±0.2	0.1 ± 0.0	0.2 ± 0.1

Table 2: Patient characteristics of the two study groups in the *in vitro* study; *This information could not be obtained in 5 subjects from the control group and 2 subjects from the caffeine group.

Variable	Control group	Caffeine group
Number	17	13
Sex (male/female)	14/3	12/1
Age (years)	66.1 ± 2.3	61.5 ± 2.4
Risk factors for atherosclerosis; number (%)		
Hypertension	9 (52.9%)	7 (53.8%)
Diabetes Mellitus	3 (17.6%)	2 (15.4%)
Hyperlipidemia	8 (47.1)	9 (69.2%)
Nicotine abuse	5 (29.4%)	6 (46.2%)
Indication for surgery		
CABG	12 (70.6%)	12 (92.3%)
Aortic valve replacement	5 (29.4%)	1 (7.7%)
Medication; number (%)		
Beta blocker	12 (70.6%)	12 (92.3%)
ACE inhibitor	5 (29.4%)	6 (46.2%)
Angiotensin II receptor antagonist	2 (11.8%)	0 (0%)
Calcium-channel blocker	8 (47.1%)	3 (23.1%)
Nitrate	9 (52.9%)	8 (61.5%)
Aspirin	11 (64.7%)	12 (92.3%)
HMG CoA reductase inhibitor	12 (70.6%)	9 (69.2%)
Insulin	1 (5.9%)	2 (15.4%)
Peri-operative medication; number (%)*		
Volatile anesthetics	3 (25%)	5 (46%)
Opioids	12 (100%)	11 (100%)

***In vivo* studies**

Plasma caffeine concentration just before ischemia was 0.2±0.1 and 6.1±0.4 mg/l in the first study and 0.1±0.1 and 6.0±0.5 mg/l in the second study after saline and caffeine infusion, respectively. In the second study, we observed an increase in systolic and diastolic blood pressure after caffeine administration (table 3).

Table 3: hemodynamic values at baseline (before caffeine/saline) and just before the ischemic exercise period (after caffeine/saline) in the group without preconditioning (the second *in vivo* study); *P<0.005 for effect of caffeine administration. †P<0.05 for comparison between effect of caffeine and effect of saline.

Group	DBP Before	DBP After	SBP Before	SBP After	HR Before	HR After
Caffeine (n=9)	73 ± 2	87 ± 3*	107 ± 3	122 ± 5*†	73 ± 4	69 ± 3
Saline (n=7)	80 ± 4	85 ± 4	113 ± 5	114 ± 4	68 ± 4	67 ± 5

^{99m}Tc-annexin A5 targeting in the thenar muscle at 1 and 4 hours after reperfusion is shown in figure 2.

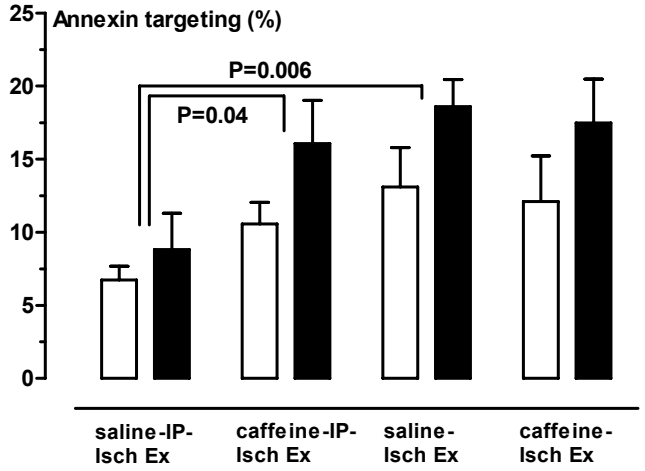

Figure 2: Annexin A5 targeting in the thenar muscle at 1 (open bars) and 4 (filled bars) hours after reperfusion in the different groups (expressed as % difference between experimental and control hand). P-values indicate differences between groups as assessed by repeated measures ANCOVA with time (t=1 and t=4 hours post-reperfusion) as within-subject factor, group (as indicated on the X-axis) as between-subject factor, and workload as covariate.

When comparing the saline groups of both studies, IP reduced annexin targeting from 13±3 % to 7±1% at 1 hour, and from 19±2% to 9±3% at 4 hours after reperfusion (P=0.006, repeated measures ANCOVA). In the first randomized study, caffeine significantly reduced the effect of

preconditioning: annexin targeting was 11±2% and 16±3% at 1 and 4 hours (P=0.04 versus saline-IP-Isch Ex). In figure 3, representative scintigraphic images of the caffeine-IP-Isch Ex and saline-IP-Isch Ex group are shown. However, in the second randomized study, caffeine did not affect annexin targeting after only the ischemic exercise procedure (13±3% at 1 hour and 19±2% at 4 hours for saline-Isch Ex and 12±3% and 18±3% for caffeine-Isch Ex; P=0.8).

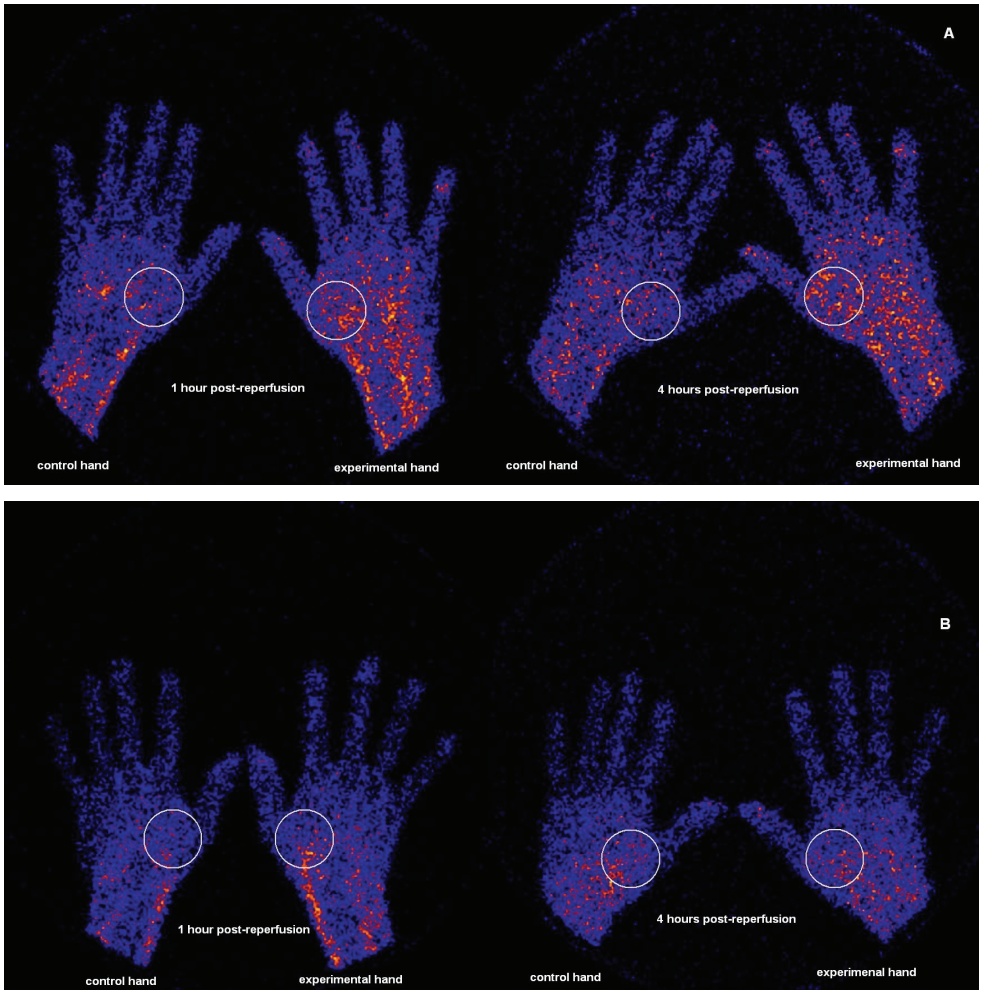

Figure 3: Representative scintigraphic images of the experimental and control hands at 1 and 4 hours after reperfusion in preconditioned subjects pretreated with caffeine (a) and saline (b). Annexin targeting was 2% and 3% after saline administration (Saline-IP-Isch Ex) and 19% and 27% after caffeine administration (Caffeine-IP-Isch Ex) at 1 and 4 hours, respectively.

In vitro study

Baseline contractile force was similar for all groups of trabeculae (table 4; P=0.7, one way ANOVA).

Table 4: baseline contractile force of the different groups of trabeculae (mean ± SE).

Group	Baseline force (g).
Control, IP	0.50±0.05
Control, no IP	0.47±0.05
Caffeine, IP	0.57±0.06
Caffeine, no IP	0.52±0.05

After 90 minutes of simulated ischemia and 120 minutes of reperfusion, only a partial recovery of contractile force was observed (figure 4).

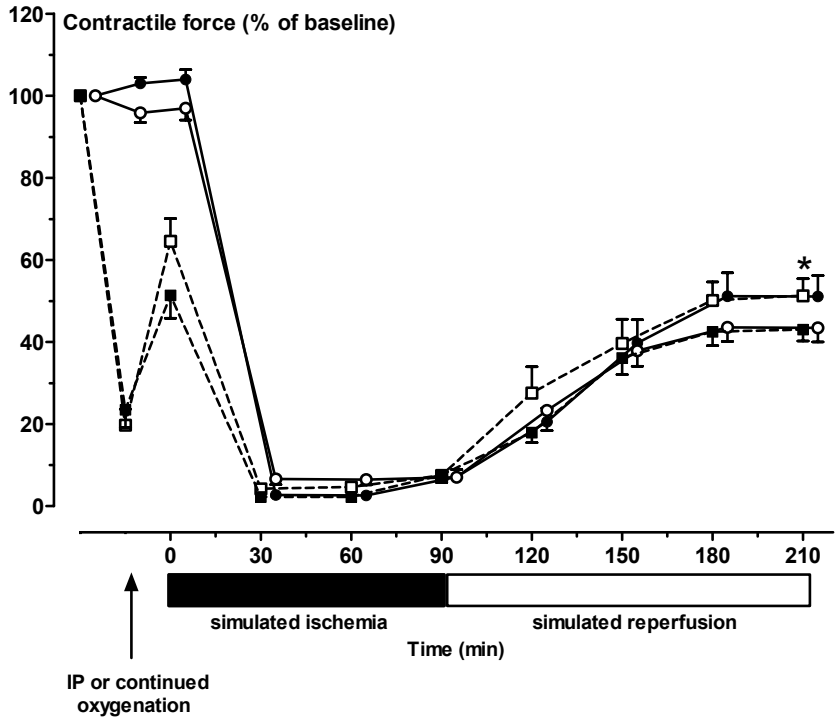

Figure 4: The course of contractile force (expressed as percentage of baseline force) in time in control trabeculae (n=17) with (open squares) and without (open circles) preconditioning and in caffeine pretreated trabeculae (n=13) with (filled squares) and without (filled circles) preconditioning. *P=0.008 for the effect of preconditioning on post-ischemic recovery of contractile force in the saline group. For the sake of clarity, the symbols representing the non-preconditioned groups are slight shifted to the right.

Ischemic preconditioning improved recovery from 43±3% to 51±4% (P=0.008), from 45±4% to 56±7% (P=0.055), and from 46±4% to 56±5% (P=0.017) for developed force, systolic, and diastolic function, respectively.
In the presence of caffeine, IP changed recovery in contractile function from 51±5% to 43±3% (P=0.10), from 51±5% to 44±3% (P=0.20), and from 54±5% to 48±3% (P=0.20) for developed force, systolic, and diastolic function, respectively. Thus, in the presence of caffeine, IP did no longer improve recovery of function. If anything, a tendency to the contrary was observed. The effect of IP on recovery of contractile force differed significantly between the control group and the caffeine group (P=0.003).

DISCUSSION

Caffeine is one of the most widely consumed pharmacologically active compounds worldwide, mainly derived from dietary sources such as coffee and tea (115). Daily intake of caffeine in US consumers is estimated at 4 mg/kg per day, which is even higher in some European countries (114). In this study, we demonstrated that administration of caffeine, in a dose comparable to this estimated daily dose, abolishes protection from IP in two experimental models in humans.

Traditionally, IP is defined as myocardial infarct size reduction induced by a preceding brief period of ischemia and reperfusion (122). In humans, however, this histological endpoint cannnot be used experimentally to study IP. Therefore, several surrogate endpoints of ischemia-reperfusion injury have been developed to study IP in humans in an experimental setting. In the present study, we used two different, complementary, and previously validated experimental models to study the effect of caffeine on IP in humans.

The *in vivo* part of our study is based on the specific and high-affinity binding of annexin A5 to phosphatidylserine residues on cellular membranes. It is well documented that loss of membrane asymmetry is an early general feature of apoptosis, which results in externalization of phosphatidylserine residues in affected cells, thus providing binding sites for annexin A5 (134, 546). By labeling recombinant annexin A5 with ^{99m}Technetium, it is possible to detect these cellular changes *in vivo* with a gamma camera. Previously, this method has been used to visualize apoptotic cells in murine and human hearts *in vivo* (137, 569). Concomitant use of conventional methods for detection of apoptosis revealed that, indeed, annexin A5 specifically binds to cells with apoptotic morphology and co-localizes with the presence of activated caspase-3 (136). Also, annexin A5 targeting could be diminished by pretreatment with caspase inhibitors (569). Because IP reduces myocardial ischemic injury in part by inhibition of apoptosis (278), we used ^{99m}Tc-annexin A5 scintigraphy as a model to study IP in humans. We demonstrated previously that ten minutes of ischemia of the nondominant forearm combined with isometric handgrip exercise (ischemic exercise) increases targeting of annexin A5 to the thenar muscle, expressed as difference in ^{99m}Tc-activity between the experimental and control hand (140). This increased targeting could be prevented by a preconditioning stimulus of ten minutes ischemia and reperfusion before ischemic exercise. Similarly, pretreatment with adenosine (administered into the brachial artery) (140) and the nucleoside uptake inhibitor dipyridamole (orally, twice daily for one week) (146) reduced annexin targeting.

In the present *in vivo* studies, we confirmed that the preconditioning stimulus reduced annexin A5 targeting induced by ischemic exercise. In the first randomized trial, pretreatment with caffeine significantly reduced the effect of IP on annexin targeting. In fact, annexin targeting in preconditioned subjects pretreated with caffeine was similar to the targeting in control subjects who were not preconditioned, indicating that the beneficial effect of IP is completely abolished. In the second randomized trial, administration of caffeine did not affect annexin targeting after ischemic exercise without preconditioning, indicating that caffeine selectively inhibits the effect of preconditioning on annexin targeting.

To confirm that these effects of caffeine are also present in myocardial tissue, we performed an additional *in vitro* study on human atrial trabeculae, obtained during heart surgery, using recovery of contractile function as endpoint of ischemia-reperfusion injury. This model has been used in several previous studies, which consistently demonstrated that preconditioning increases post-ischemic recovery of contractile function (390, 391, 440, 446, 472). Also, these studies showed that preconditioning in this model is dependent on stimulation of opioid receptors and protein kinase C, and opening of K_{ATP}-channels (390, 440), and can be mimicked by stimulation of adenosine A_1 and A_3 receptors, comparable to studies using histological infarct size as endpoint of ischemia-reperfusion injury (86, 87, 314, 570, 571). It has to be realized that recovery of mechanical function after ischemia is influenced by both the number of surviving myocytes and the effect of stunning of these myocytes. The effect of preconditoning on stunning is much more controversial than its effect on cellular death (272). However, because in the present model of 90 minutes of ischemia an effect of preconditioning has consistently been demonstrated, cellular death probably plays a major role in determining functional outcome.

In our study, the effect of IP was less than in previous reports from other groups (390, 391). Interestingly, this is completely caused by the lower functional recovery of control trabeculae in these previous reports. Differences in experimental set up, patient group and peri-operative management could possibly account for this difference. For example, all patients included in the present study received opioid receptor agonists and approximately one-third of patients received volatile anesthetics before resection of the atrial tissue. Because these drugs mimic IP (448), this may have resulted in improved functional recovery in the control trabeculae compared with that in previous studies, leaving less space for additional protection by IP. Unfortunately, in these previous studies, peri-operative medication use was not mentioned. In the present study, post-ischemic recovery of contractile function was significantly increased by preconditioning in the saline group. In contrast, in the caffeine group IP did not potentiate recovery of contractile function. If anything, a reduction of functional recovery was observed in the preconditioned trabeculae in the presence of caffeine. Interestingly, recovery of contractile force in the non-preconditioned trabeculae was higher in the caffeine group, although this was not significant (P=0.2). Probably, this reflects a transient positive inotropic effect of caffeine, which is observed as long as caffeine is present. Indeed, in the subset of trabeculae without preconditioning, we observed a steady increase in contractile force in the 20 minutes immediately after administration of caffeine compared with that in the control trabeculae (data not shown). This is in concordance with previous studies

demonstrating positive inotropic effects of xanthine derivatives (572, 573). This could be caused by antagonism of the negative inotropic effect of endogenous adenosine, which is continuously produced by cardiomyocytes, although it is suggested that alternative mechanisms might also be involved (574). However, inhibition of phosphodiesterase and mobilization of intracellular calcium is induced only at higher caffeine concentrations than the concentration achieved in our study (115). This positive inotropic effect of caffeine is also present in the preconditioned trabeculae, so any beneficial effect of preconditioning should have increased recovery of contractile force compared with the recovery without preconditioning.

The plasma caffeine concentration immediately before the initiation of forearm ischemia averaged approximately 6 mg/l in the *in vivo* studies and was slightly higher in the *in vitro* study. This concentration occurs in daily life after an intake of 2 to 4 cups of coffee (575, 576). At this concentration, the pharmacological actions of caffeine are solely due to binding to adenosine receptors and antagonism of the actions of endogenous adenosine. Other mechanisms of action of caffeine, such as inhibition of phosphodiesterase or calcium release from intracellular stores, occur at higher concentrations (115).

Impairment of the powerful infarct size-limiting effect of IP by caffeine would theoretically cause an association between caffeine use (especially derived from coffee consumption) and clinical endpoints of ischemia-reperfusion injury. However, the association between coffee intake and cardiovascular disease remains controversial, although for subjects consuming more than five cups of coffee daily there seems to be an increased risk for cardiovascular disease (577, 578). Interestingly, a recent study showed that among patients with a CYP1A2 genotype predicting slow caffeine metabolism, intake of coffee (even 2 to 3 cups daily) was associated with an increased risk of nonfatal myocardial infarction, suggesting that caffeine does play a role in this association (579). When considering these epidemiological trials in the light of our present results, it has to be realized that inhibition of preconditioning will not increase the incidence of cardiovascular events *per se*, but rather would be expected to adversely affect the outcome after myocardial infarction (i.e. the incidence of cardiac failure or death) in the subset of patients experiencing pre-infarction ischemia. This potential effect has generally not been evaluated in studies on the association of coffee consumption and the incidence of cardiovascular disease. Also, our study refers to the acute effects of a single dose of caffeine. This does not necessarily hold for chronic use, especially because tolerance to the pharmacological effects of caffeine has been described (580). However, several publications have shown that a considerable fraction of the population does not develop complete tolerance to caffeine despite the daily use of caffeinated products (581, 582).

In conclusion, the present study shows that caffeine, at a concentration reached in daily life after drinking 2 to 4 cups of coffee, impairs the protection afforded by ischemic preconditioning in two experimental models in humans. This observation provides an experimental basis to study the effects of caffeine consumption on cardiovascular morbidity and mortality in patients who are at increased risk for ischemic events.

ACKNOWLEDGEMENTS

NP Riksen is a recipient of a fellowship of the Netherlands Organization of Scientific Research (ZonMw). The contribution of GA Rongen has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. Additional financial support was obtained from the European Union (project number: QLK1-CT-2000-00069). The NAS 2020 kits for preparation of ^{99m}Tc-HYNIC-rh-Annexin A5 were donated by Dr. JL Vanderheyden, Theseus Imaging Corparation, Boston, MA, USA. We would like to thank Petra van den Broek for the determination of caffeine concentrations. The investigators appreciate the support from the staff of the Clinical Research Center Nijmegen.

CHAPTER 4.6

METHOTREXATE MODULATES THE KINETICS OF ADENOSINE IN HUMANS *IN VIVO*

Ann Rheum Dis 2006; 65:465-70

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ABSTRACT

Objective Animal studies suggest that the anti-inflammatory effect of methotrexate (MTX) is mediated by increased adenosine concentrations. We aimed to assess the effect of MTX on the vasodilator effects of adenosine and the nucleoside uptake inhibitor dipyridamole in humans *in vivo* as a marker for changes in adenosine kinetics.

Methods Ten patients with active arthritis were treated with MTX (15 mg/week). Measurements were performed before and after 12 weeks of treatment. At these timepoints, activity of adenosine deaminase was measured in isolated lymphocytes, and forearm blood flow (FBF) was determined by venous occlusion plethysmography during administration of adenosine and dipyridamole into the brachial artery.

Results The V_{max} of adenosine deaminase in lymphocytes was reduced by MTX treatment ($P<0.05$). MTX significantly enhanced the vasodilator response to adenosine (0.5 and 1.5 μg/min per dl of forearm tissue; FBF ratio increased from 1.2 ± 0.2 to 1.4 ± 0.2 and 2.2 ± 0.2, respectively, before and from 1.3±0.1 to 1.8±0.2 and 3.2±0.5 during MTX treatment, mean ± SE; $P<0.05$). Also, dipyridamole-induced vasodilation (30 and 100 μg/min/dl) was enhanced by MTX (FBF ratio increased from 1.2 ± 0.2 to 1.5 ± 0.3 and 1.8 ± 0.2, respectively, before and from 1.3 ± 0.1 to 1.8 ± 0.2 and 2.4 ± 0.4 during MTX treatment; $P<0.05$).

Conclusions MTX treatment inhibits deamination of adenosine and potentiates adenosine-induced vasodilation. Also, dipyridamole-induced vasodilation is enhanced by MTX treatment, suggesting an increased extracellular formation of adenosine. These effects on the adenosine kinetics in humans may contribute to the therapeutic efficacy of MTX.

Key Words: Methotrexate, Adenosine, Blood flow.

INTRODUCTION

Because of its favourable efficacy and toxicity profile, methotrexate (MTX) is often the first disease modifying antirheumatic drug (DMARD) prescribed in patients with rheumatoid arthritis (583). The mechanism of action of MTX remains a subject of controversy, although it is generally accepted that this is different from the anti-proliferative effect of high-dose MTX treatment in the treatment of neoplasms (584, 585). In the past decade, animal studies have provided several lines of evidence that the anti-inflammatory effect of MTX is mediated by adenosine receptor stimulation (584).

Adenosine is a purine nucleoside that is formed both intra- and extracellularly by degradation of adenosine mono-phosphate (AMP) (figure 1) (6). Degradation of adenosine by the enzymes adenosine deaminase and adenosine kinase, however, is confined to the intracellular compartment. As such, facilitated transport of adenosine over the cellular membrane is mainly directed from outside the cell inwards (50). There are four types of G-protein-coupled adenosine receptor, which are designated A_1, A_{2A}, A_{2B}, and A_3 receptors (6). Stimulation of the A_{2A} receptor potently inhibits inflammation (10), and induces vasodilation (5).

In humans, evidence for a role of adenosine is either indirect (586, 587) or controversial (588-592). This is mainy due to methodological shortcomings and the highly complicated determination of the adenosine concentration (51).

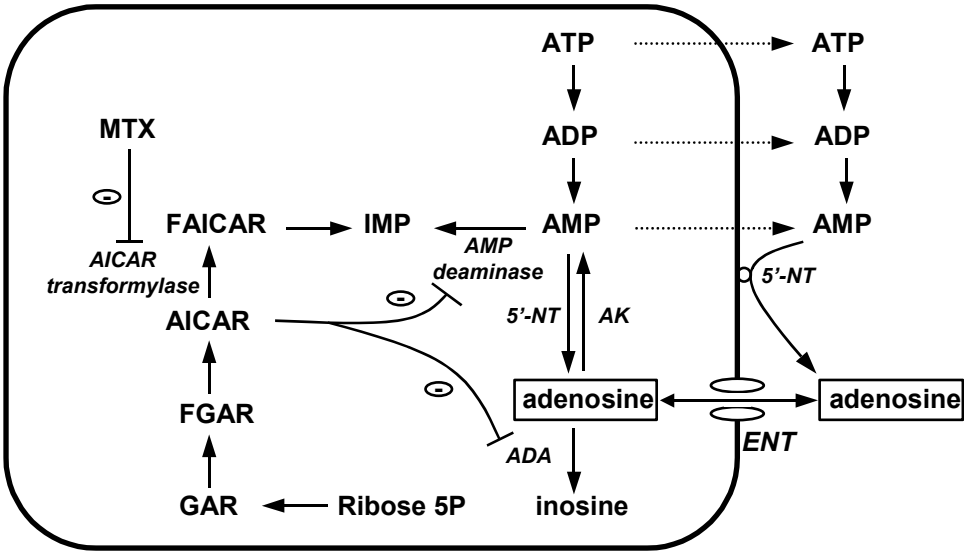

Figure 1: Simplified representation of the effect of methotrexate on adenosine metabolism. Polyglutamated methotrexate inhibits AICAR transformylase, which results in the intracellular accumulation of AICAR, which inhibits adenosine deaminase and AMP deaminase. Consequently, irreversible degradation of adenosine to inosine is inhibited as well as the conversion of AMP in IMP. Subsequently, AMP is extracellularly converted to adenosine by the ecto-5'-nucleotidase.

AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide; ENT: equilibrative nucleoside transporter; FAICAR: 10-formyl AICAR; FGAR: 10-formyl GAR; GAR: glycinamide ribonucleotide; IMP: inosine mono-phosphate; 5'-NT: 5'-nucleotidase.

In this study, we aimed at assessing whether adenosine receptor stimulation is indeed increased by MTX treatment in humans *in vivo*. We avoided the methodological problems of measuring adenosine concentrations by determining the vasodilator response to adenosine and dipyridamole, reflecting alterations in degradation and formation of adenosine, respectively. Dipyridamole is a potent inhibitor of the equilibrative nucleoside transporter (figure 1)(593) and, consequently, increases extracellular adenosine, and induces vasodilation, at a rate proportional to extracellular formation of adenosine. Finally, we also determined the activity of adenosine deaminase in lymphocytes and erythrocytes to explore the mechanism of the altered adenosine kinetics by MTX.

PATIENTS AND METHODS

Subjects

Adult outpatients with active arthritis in whom MTX treatment was indicated according to their treating rheumatologist were asked to participate. Concomitant use of other DMARD's, non-steroidal anti-inflammatory drugs (NSAID's) or corticosteroids was not allowed to be changed from one month before treatment initiation until the end of the study. Exclusion criteria were pregnancy, breast-feeding, asthma, alcohol abuse (> 20 units/week), elevated liver enzymes (alanine aminotransferase - ALT - > 3 times the upper limit), renal insufficiency (estimated clearance <50 ml/min), thrombocytopenia (<120×10^9/l), or leukocytopenia (<3.5 ×10^9/l). Patients were not allowed to enter the study if they had been treated previously with MTX, or if they were treated currently with sulfasalazine, dipyridamole, folic acid or folinic acid. Folic acid supplementation was not given in the 12-week treatment period to avoid any possible interference with our measurements.

The study protocol was approved by the Institutional Review Board of the Radboud University Nijmegen Medical Center and the investigation conforms with the principles outlined in the Declaration of Helsinki. Ten patients agreed to participate and signed written informed consent before participation. Seven patients were diagnosed with rheumatoid arthritis, two patients with psoriatic arthritis and one patient with an unspecified oligoarthritis. Other baseline characteristics are shown in table 1.

Table 1: Baseline characteristics of the patients.

Variable	Mean ± SD
Sex (m/f)	5 / 5
Age (years)	53.2 ± 12.0
Disease Activity Score (DAS)	4.0 ± 1.1
Body Mass Index (kg/m^2)	24.7 ± 5.4
Creatinine (μmol/l)	88.0 ± 56.4
Cholesterol (mmol/l)	5.4 ± 0.6
Plasma glucose (mmol/l)	5.3 ± 0.5

Experimental Protocol

All patients started oral treatment with MTX at a dose of 15 mg per week during the study period. Vasodilator effects of adenosine and dipyridamole were assessed before and 12 weeks after the

start of the treatment, 2 hours after the intake of the weekly MTX dose. All experiments were performed in the morning in a temperature-controlled laboratory (23 °C). Participants were asked to abstain from caffeine-containing products for at least 24 hours before each experiment because caffeine is an effective adenosine receptor antagonist (116). NSAID's were discontinued for at least 24 hours before each experiment to avoid any influence of cyclooxygenase inhibition on vascular function.
On these visits the disease activity score (DAS) (594) was obtained by a rheumatologist (PB), and blood was drawn for determination of ALT, alkaline phosphatase (AF), creatinine, total blood count, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), total plasma homocysteine and the activity of the adenosine deaminase enzyme in erythrocytes and lymphocytes.

After local anesthesia (xylocaine 2%) the brachial artery of the non-dominant arm was cannulated with a 20-gauge catheter (Angiocath, Deseret Medical, UT, USA) for intra-arterial drug administration (automatic syringe infusion pump, type STC-521, Terumo Corporation, Tokyo, Japan) and blood pressure recording (Hewlett Packard GmbH, Böblingen, Germany). Forearm blood flow (FBF) was registered simultaneously on both arms by venous occlusion plethysmography using mercury-in-silastic strain-gauges (Hokanson EC4, Hokanson, Inc., Washington, USA), as previously described (141). Each drug dose was infused for 5 minutes and FBF was recorded three times per minute during the last 2 minutes of each infusion. Before each drug infusion, a wrist cuff was inflated to 200 mmHg to exclude the hand circulation from the measurements.
Baseline FBF was assessed during infusion of saline into the brachial artery. Subsequently adenosine was infused in a dose of 0.5 and 1.5 μg/min per dl of forearm tissue. After 30 minutes baseline FBF was measured again during infusion of saline and finally, dipyridamole was infused in a dose of 30 and 100 μg/min/dl.

Analytical methods
Total plasma homocysteine was determined by use of reversed-phase HPLC, as previously described (213). For the determination of adenosine deaminase activity, erythrocytes were isolated from freshly-drawn blood by centrifugation, washed two times in saline and resuspended in MOPS buffer (10 mM MOPS, 0.9% NaCl, pH 7.4) to obtain a 20% (v/v) solution. Subsequently, the erythrocytes were lysed by adding 6 volumes of cold distilled water for 5 minutes. After centrifugation for 10 minutes at 4 °C, the supernatant was stored at –70 °C until analysis. Lymphocytes were isolated by Ficoll-Paque centrifugation and were lysed with M-PER® Mammalian protein extraction reagent in the presence of Halt™ protease inhibitor cocktail and EDTA solution. After incubation for 10 minutes (22 °C), and centrifugation for 15 minutes, the supernatant was stored at –70 °C until analysis.
For determination of adenosine deaminase activity, adenosine was added to erythrocyte lysate in a final concentration of 0, 25, 50, 100, 200 and 300 μmol/l at 37 °C. Each 200 μl of incubation mixture contained 25 μl of cellular lysate and 50 mM of Tris-HCl (pH 7.4). After 15 minutes the reaction was stopped by the addition of 50 μl 1.5 M $HClO_4$ followed by centrifugation (3 minutes). Subsequently, 125 μl of the supernatant was mixed with 125 μl trioctylamine in chloroform, and after

centrifugation (3 minutes) 50 µl of the neutralized upper layer was used for HPLC analysis of inosine and hypoxanthine with UV-detection.

Drugs and solutions

Solutions of adenosine (Adenocor, Sanofi-Synthelabo, Maassluis, the Netherlands) and dipyridamole (Persantin, Boehringer Ingelheim, Espana S.A., Spain) were freshly prepared before each experiment with saline as solvent.

Data analysis

All data are shown as mean ± SD unless stated otherwise. For each patient, V_{max} and K_m values of adenosine deaminase were calculated according to Michaelis-Menten kinetics (GraphPad Prism 4 for Windows). Activity was related to the total protein content in lymphocytes and to the protein content of the membranous fraction in erythrocytes, as determined by the Lowry assay. The effect of MTX treatment on laboratory values and disease activity scores was calculated with a Wilcoxon signed ranks test as not all variables showed a Gaussian distribution.

FBF was measured in both arms simultaneously and the ratio of the FBF in the experimental arm to the control arm (FBF ratio) was calculated to adjust for random changes unrelated to the local stimulus (595). The effect of MTX treatment on the vasodilator response to adenosine and dipyridamole was calculated with an analysis of variance for repeated measures (SPSS for windows, release 12.0.1).

RESULTS

One patient was excluded from analysis because MTX treatment had to be discontinued as a result of a rise in ALT activity during the study. Disease activity score was not significantly decreased by MTX (from 4.0 ± 1.1 to 3.2 ± 2.0, n=9, *P>0.1*). Plasma concentrations of CRP and Hb tended to decrease during MTX treatment (table 2, *P*=0.1). ALT activity and total plasma homocysteine concentration were significantly increased by MTX treatment (table 2). Other biochemical or hemodynamic parameters did not change significantly.

Table 2: Laboratory values and hemodynamic measures. **P*<0.01 for the effect of methotrexate.

Variable	Before methotrexate	During methotrexate
ALT (U/l)	20 ± 11	45 ± 24*
Creatinine (µmol/l)	88 ± 56	83 ± 46
CRP (mg/l)	24 ± 48	10 ± 15
ESR (mm/h)	15 ± 18	16 ± 22
Hb (mmol/l)	8.1 ± 0.8	7.7 ± 1.1
Leucocytes (× 10^9/l)	7.8 ± 2.8	7.1 ± 2.4
Thrombocytes (× 10^9/l)	253 ± 92	278 ±88
SBP (mmHg)	142 ± 20	139 ± 18
DBP (mmHg)	70 ± 7	70 ± 9
Heart rate (bpm)	65 ± 12	66 ± 13
Homocysteine (µmol/l)	10.6 ± 4.1	14.9 ± 4.9*

In lymphocytes, but not in erythrocytes, the V_{max} value of the enzyme adenosine deaminase was significantly decreased by MTX treatment, whereas K_m values were not significantly affected (table 3).

Table 3: adenosine deaminase activity. V_{max} values expressed as nmol/min/mg protein; K_m values expressed as µmol/l.*P<0.05 for the effect of methotrexate. P>0.1 for other parameters.

Variable		Before methotrexate	During methotrexate
Lymphocytes	V_{max}	20.7 ± 5.6	16.2 ± 6.0*
	K_m	40.7 ± 2.9	38.3 ± 3.6
Erythrocytes	V_{max}	100.9 ± 31.7	111.3 ± 42.3
	K_m	43.9 ± 6.9	42.6 ± 5.5

Baseline FBF in the experimental arm was similar on both occasions (2.9 ± 0.9 ml/min/dl before and 2.9 ± 0.8 during MTX treatment, P>0.1). Also on both occasions, FBF completely returned towards baseline levels after the 30-minute period between the adenosine and dipyridamole infusions and MTX treatment did not significantly affect FBF in the control arm (data not shown). MTX treatment resulted in a significant enhancement of both adenosine-induced vasodilation and dipyridamole-induced vasodilation (P<0.05; figure 2).

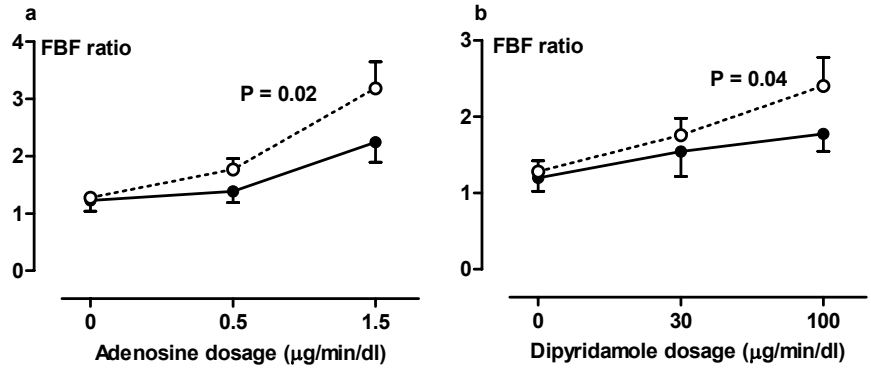

Figure 2: Forearm vasodilation (mean ± SE) induced by the infusion of adenosine (a) and dipyridamole (b) into the brachial artery before initiation of MTX treatment (filled squares) and after 12 weeks of treatment (open squares). P-values denote the results of analysis of variance for repeated measures.

DISCUSSION

In this study we demonstrated in humans *in vivo* that MTX treatment inhibits adenosine deaminase. Also, adenosine-induced forearm vasodilation was significantly potentiated by MTX. This might be due to the decreased intracellular deamination of adenosine, because it was

previously shown that adenosine deamination rather than transport of adenosine over the cellular membrane is rate-limiting for the overall catabolism of adenosine (567). We also showed that dipyridamole-induced vasodilation is potentiated by MTX treatment. This observation indicates that, besides inhibition of intracellular degradation, also extracellular formation of endogenous adenosine is increased, which is compatible with previous *in vitro* findings (596). In conclusion, we demonstrated for the first time in humans *in vivo* that treatment with MTX potentiates adenosine A_{2A} receptor mediated effects.

The effect of MTX on the metabolism of adenosine was first described by Cronstein *et al ex vivo* and in animal experiments (597). They showed that in isolated human fibroblasts and endothelial cells, pretreatment with MTX increases extracellular adenosine. Recently, it was demonstrated that in a rat model of adjuvant arthritis, adenosine receptor antagonists completely abolish the anti-inflammatory effects of MTX (598). Moreover, in adenosine A_{2A} and A_3 receptor knock-out mice, MTX treatment no longer induces any anti-inflammatory effects (599).

Human *in vivo* data on the potential role of adenosine in the mechanism of action of MTX are scarce, controversial, and indirect. Two recent reports suggest that a high intake of caffeinated products diminishes the anti-rheumatic effect of MTX (587). As caffeine is an effective adenosine receptor antagonist already at concentrations reached after regular coffee consumption (115), this is consistent with a role for adenosine receptor stimulation in the mechanism of action of MTX. Interventional studies which determined adenosine concentration before and after administration of MTX provided controversial results and most studies showed negative results (588-592). An important reason for these inconsistent and mostly negative results is the duration of MTX treatment. It is necessary to administer MTX for several days to weeks to permit polyglutamation and intracellular accumulation of AICAR, which is required for enhanced adenosine formation (599). In contrast, in most previous studies, adenosine concentrations were determined after at most one day (589-591) or a few days (588). Also, reliable measurement of adenosine is highly cumbersome as its halflife is less than one second and the endothelium constitutes an active metabolic barrier for adenosine, resulting in a functional compartmentalization of adenosine (51, 52). In the present study, we administered MTX for 12 weeks and bypassed the methodological diffulties of adenosine determination by using adenosine- and dipyridamole-induced vasodilation as a reflection of adenosine degradation and formation, respectively.

The mechanism by which MTX affects the kinetics and dynamics of adenosine has partially been elucidated by previous animal studies. Most probably, MTX interferes with the *de novo* purine synthesis pathway in cells (585, 600). Polyglutamates of MTX, the long-lived intracellular metabolites of MTX, are potent competitive inhibitors of the enzyme 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, resulting in the intracellular accumulation of AICAR (600) (Figure 1). In turn, it has been shown *in vitro* that AICARiboside inhibits catalytic activity of adenosine deaminase (600, 601), and AICARibotide inhibits AMP deaminase (600). Recently, Morabito *et al* showed that the anti-inflammatory effects of MTX can be abolished by inhibition of ecto-5′-nucleotidase (596), emphasizing the importance of extracellular conversion of AMP to adenosine. It was suggested that inhibition of AMP deaminase promotes release of adenine

nucleotides, by an as yet unidentified mechanism, which are converted by extracellular 5'-nucleotidase to adenosine (figure 1).

Our study provides additional mechanistic insight, which links up nicely with the abovementioned previous findings. It was previously shown in humans that MTX treatment decreases the activity of adenosine deaminase. However, effects of MTX on the V_{max} and K_m values of this enzyme were not assessed (602). The present study showed that MTX decreases the V_{max} but not the K_m of adenosine deaminase in lymphocytes. Unfortunately, from these results it cannot be concluded whether this observed change is due to direct non-competitive enzyme inhibition or decreased enzyme levels, or whether this reflects changes in lymphocyte subpopulations, which could differ in their adenosine deaminase activity (603). To provide further evidence that MTX modulates adenosine metabolism by interfering with *de novo* purine synthesis, we also determined activity of adenosine deaminase in erythrocytes, a cell type that lacks the capacity for *de novo* purine synthesis (604). Indeed, in these cells we observed no change of activity of adenosine deaminase during MTX treatment. Unfortunately, we were not able to assess enzymatic activity of other enzymes involved in purine metabolism, such as AMP deaminase and ecto-5'-nucleotidase. It has to be realized that erythrocytes are responsible for the bulk of breakdown of circulating adenosine and that, therefore, adenosine deamination in lymphocytes probably only plays a minor role in the observed increased adenosine-induced vasodilation in our study. However, endothelial cells and vascular smooth muscle cells contribute significantly to adenosine metabolism (55). We postulate that in these cells, adenosine deaminase activity is also reduced, resulting in an increased adenosine concentration able to stimulate adenosine receptors on these cells. In contrast to the breakdown of circulating adenosine, lymphocytes might play an important role in the regulation of adenosine concentration in areas of inflammation. Therefore, the decreased adenosine deaminase activity in lymphocytes could contribute to the anti-inflammatory effect of MTX.

Additional mechanistic information could be obtained from the *in vivo* part of this study. As dipyridamole prevents cellular uptake of endogenous adenosine, it increases the extracellular concentration of adenosine at a rate that is proportional to extracellular formation of adenosine. We have demonstrated previously that dipyridamole-induced vasodilation in humans is indeed due to inhibition of cellular adenosine uptake: dipyridamole potentiates the vasodilator response to adenosine (154), dipyridamole increases the adenosine concentration in the forearm venous effluent during administration of adenosine into the brachial artery (153), and dipyridamole-induced vasodilation (100 μg/min/dl) is inhibited by the adenosine receptor antagonist theophylline (106). The present study shows that dipyridamole-induced vasodilation is significantly enhanced during MTX treatment. This finding translates the previous *in vitro* finding of increased extracellular dephosphorylation of AMP during treatment with MTX (596) to the human *in vivo* situation.

The present study revealed that MTX treatment potentiates the vasodilator effect of adenosine. It has to be realized that adenosine receptor stimulation not only inhibits inflammation and induces vasodilation, but that it initiates various cardiovascular effects, such as negative inotropic and chronotropic cardiac effects, presynaptic inhibition of sympathetic neurotransmitter release, inhibition of vascular smooth muscle cell proliferation, and inhibition of thrombocyte aggregation

(5). Also, adenosine receptor stimulation renders the myocardium more resistant to ischemia and reperfusion injury (8). Taken together, these effects have the potential to protect the heart during ischemia and prevent or slow down the process of atherosclerosis. Patients with rheumatoid arthritis have a higher incidence of cardiovascular disease than the general population (605, 606). Interestingly, a recent study suggested that the beneficial effect of MTX on cardiovascular mortality is superior to that of other antirheumatic drugs (607), although another study did not find this benefit (608). When considering the abovementioned cardiovascular effects of adenosine, one could easily appreciate that increased adenosine receptor stimulation could be responsible for this beneficial cardiovascular effect of MTX. Indeed, it was shown previously in canine hearts that MTX limits myocardial infarct size via adenosine-dependent mechanisms (609).

Finally, potential limitations of the present study need to be discussed. It has to be realized that, due to the use of a clinical study population in need of immediate treatment, the design of the present study was open-labeled and non-randomized. In healthy volunteers a more sophisticated design would have been possible, but in our opinion it is unethical to administer MTX to healthy volunteers for a long time. We assured that the use of other (anti-inflammatory) drugs was kept constant during the study period, in order to prevent confounding by other drugs. Finally, plasma homocysteine concentration needs to be considered as determinant of adenosine-induced vasodilation. MTX treatment increases plasma homocysteine concentration by interfering with folate-dependent remethylation of homocysteine (610). Any increase in plasma homocysteine, in turn, could stimulate synthesis of S-adenosylhomocysteine at the expense of free adenosine, as we previously described (159, 206). Also, hyperhomocysteinemia induces endothelial dysfunction (611). Fortunately, these potential effects of homocysteine on vascular reactivity are opposite to our present findings, and therefore our conclusion would even be more convincing if we had have been able to correct for the rise in homocysteine.

Our study adds important human *in vivo* data to the growing body of evidence that adenosine is an important mediator of the therapeutic efficacy of MTX in patients with rheumatoid arthritis. These insights provide potential alternative targets for pharmacological intervention in these patients, such as adenosine uptake inhibition. Dipyridamole, either alone or as additive to MTX, also increases extracellular endogenous adenosine and, thus, would also be expected to suppress inflammation in this patient group. To our knowledge, this potential anti-inflammatory effect of dipyridamole has never been systematically studied in a clinical population.

ACKNOWLEDGEMENTS

Niels P. Riksen is a recipient of a fellowship of the Netherlands Organization for Scientific Research (ZonMw). Gerard A. Rongen is a fellow of the Royal Netherlands Academy of Arts and Sciences (KNAW). We would like to thank Dr. Henk Blom and Diny van Oppenraaij for the determination of homocysteine. None of the authors have any competing financial interest in this manuscript.

CHAPTER 4.7

THE NONSPECIFIC ANTI-INFLAMMATORY THERAPY WITH METHOTREXATE FOR PATIENTS WITH CHRONIC HEART FAILURE

Am Heart J 2006;151:e9

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TO THE EDITOR*:* With interest we read the article by Gong *et al*, in which they demonstrate that in patients with chronic heart failure, treatment with methotrexate (MTX) has anti-inflammatory effects and improves clinical parameters (612). We consider it important to emphasize the potential role of endogenous adenosine in these cardiovascular effects of MTX.

As the authors briefly mention in their discussion, the anti-inflammatory effects of MTX in several animal models are critically dependent on adenosine receptor stimulation (598). Indeed, adenosine is a potent immunomodulator. However, in addition, adenosine receptor stimulation induces several beneficial cardiovascular effects independent from its immunomodulation, including vasodilation, inhibition of sympathetic neurotransmitter release, and induction of ischemic preconditioning (5). Indeed, pharmacological interventions and genetic polymorphisms that increase endogenous adenosine concentrations are associated with a better outcome in patients with heart failure (112, 119).

Recently, we have demonstrated that also in humans *in vivo*, a 12-week treatment with MTX decreases activity of adenosine deaminase and potentiates adenosine-induced vasodilation. Also, vasodilation evoked by the nucleoside transport inhibitor dipyridamole was enhanced, indicating that MTX increases extracellular formation of adenosine (613). In line with these findings, it was shown in canine hearts that pretreatment with MTX reduces myocardial infarct size via an adenosine-dependent mechanism (609).

In conclusion, we hypothesize that the beneficial effects of MTX in patients with heart failure, as described in the study by Gong *et al*, are due to increased adenosine receptor stimulation. The observed change in plasma cytokine concentrations could well be an epiphenomenon, also caused by the increased adenosine concentration, rather than a mediator of the beneficial effect of MTX, because clinical trials with anticytokine therapies in these patients have not shown any clinical benefit (614).

ACKNOWLEDGEMENTS

NP Riksen is recipient of an AGIKO-fellowship of the Netherlands Organization for Scientific Research (ZonMw).

CHAPTER 4.8

IN VIVO EVIDENCE AGAINST A ROLE FOR ADENOSINE IN THE EXERCISE PRESSOR REFLEX IN HUMANS

J Appl Physiol 2005;99:522-7

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ABSTRACT

The pressor response to exercise is of great importance in both physiology and pathophysiology. Whether endogenous adenosine is a trigger for this reflex remains controversial. Muscle interstitial adenosine concentration can be determined by microdialysis. However, there are indications that local muscle cell damage by the microdialysis probe confounds these measurements in exercising muscle. Therefore, we used the nucleoside uptake inhibitor dipyridamole as pharmacological tool to bypass this confounding. We used microdialysis probes to measure endogenous adenosine in forearm skeletal muscle of healthy volunteers during 2 cycles of 15 minutes of intermittent isometric handgripping. During the second contraction, dipyridamole (12 μg/min/dl forearm) was administered into the brachial artery. Dipyridamole potentiated the exercise-induced increase in dialysate adenosine from 0.30 ± 0.08 to 0.48 ± 0.10 μmol/l (n=9, $P<0.05$), but did not potentiate the exercise-induced increase in blood pressure. A time-control study without dipyridamole revealed no difference in exercise-induced increase in adenosine between both contractions (n=8). To exclude the possibility that the dipyridamole-induced increase in dialysate adenosine originates from extravasation of increased circulating adenosine, we simultaneously measured adenosine with microdialysis probes in forearm muscle and antecubital vein. In a separate group of 9 volunteers, simultaneous intrabrachial infusion of 100 μg/min/dl dipyridamole and 5 μg/min/dl adenosine increased dialysate adenosine from the intravenous but not the interstitial probe, indicating preserved endothelial barrier function for adenosine.

We conclude that dipyridamole significantly inhibits uptake of interstitial adenosine without affecting the pressor response to exercise, suggesting that interstitial adenosine is not involved in the pressor response to rhythmic isometric exercise.

Keywords: adenosine, blood pressure, exercise, microdialysis

INTRODUCTION

The increase in arterial blood pressure resulting from strenuous exercise of skeletal muscle is known as the exercise pressor reflex (615, 616). The exercising muscle releases several metabolites, which stimulate metabosensitive afferents that trigger this reflex (616). Several studies have provided evidence that the endogenous nucleoside adenosine contributes to this metabosensitive afferent activation (79, 617-619). On the contrary, other studies challenge the role of adenosine in triggering the exercise pressor reflex and, thus, the role of adenosine remains controversial (620-625). The present study aims to explain this controversy in terms of methodological shortcomings. Because of the very short half-life of adenosine (51) and the strong endothelial barrier for adenosine (52), it has long been very difficult to measure the concentration of endogenous adenosine in the muscle interstitial compartment, where the thin-fiber afferents responsible for initiating the pressor reflex are mainly located. However, with the recently introduced microdialysis method it is possible to accurately measure endogenous interstitial adenosine in resting muscle in humans *in vivo* (209, 626). Several investigators also used microdialysis in exercising muscle and they showed that exercise increases interstitial endogenous adenosine (209, 617, 626-628). The demonstration of an association between exercise intensity, muscle interstitial adenosine concentration and increase in muscle sympathetic nerve activity (MSNA) strongly favors a role for adenosine in triggering the pressor reflex. However, there are indications that measurements of adenosine with microdialysis in exercising muscle are confounded by mechanical damage of muscle cells located in the vicinity of the probe (629). Consequently, the increased dialysate adenosine concentration during exercise does not necessarily reflect a generalized increase in muscle interstitial adenosine but rather a local increase by probe-related muscle cell injury.

We performed the present study to directly study the role for adenosine as trigger of the exercise pressor reflex with the microdialysis method, but we bypassed the possible confounding of local muscle cell damage by using the nucleoside uptake inhibitor dipyridamole as pharmacological tool to increase the interstitial adenosine concentration. We found that dipyridamole potentiates the exercise-induced increase in dialysate adenosine, indicating significant interstitial adenosine uptake inhibition, but not the exercise-induced pressor response. To exclude that the dipyridamole-induced increase in dialysate adenosine originates from extravasation of increased circulating adenosine, we performed an additional series of experiments with simultaneous measurements of interstitial and circulating adenosine.

METHODS

The study was approved by the Institutional Review Board of the Radboud University Nijmegen Medical Center, and each subject gave written informed consent before participation.

Subjects

The baseline characteristics of the 26 participants of the different study protocols are shown in table 1.

Table 1: baseline characteristics of the study groups (mean ± SD)

	Protocol 1: Dipyridamole	Protocol 1: Time control	Protocol 2
Number (M/F)	9 (1/8)	8 (5/3)	9 (5/4)
Age (years)	20.5 ± 1.7	21.0 ± 1.2	23.7 ± 2.8
BMI (kg/m²)	22.9 ± 2.3	21.9 ± 2.6	22.9 ± 2.1
SBP (mmHg)	120.0 ± 5.2	123.5 ± 11.4	122.7 ± 5.3
DBP (mmHg)	73.0 ± 7.8	72.8 ± 6.2	73.9 ± 8.0
Heart rate (bpm)	61.8 ± 10.8	63.5 ± 9.7	66.9 ± 9.7
Cholesterol (mmol/l)	4.6 ± 0.5	4.0 ± 0.7	4.0 ± 0.7
Triglycerides (mmol/l)	1.0 ± 0.2	0.9 ± 0.5	1.0 ± 0.4
Glucose (mmol/l)	4.5 ± 0.4	4.6 ± 0.4	4.7 ± 0.3

The subjects were normotensive nonsmokers and were not taking any medications except for oral contraceptives. The subjects underwent a physical examination, including electrocardiography, before entering the study. Subjects with a history of pulmonary or cardiovascular disease were excluded. All experiments were performed after at least 24 hours of caffeine abstinence and at least 2 hours of food abstinence.

Experimental Protocol

The experiments started in the morning in a temperature-controlled laboratory (23°C), with the subjects in supine position. The brachial artery of the non-dominant arm was cannulated for drug infusion and blood pressure recording, as previously described (141). In the first protocol, blood pressure was measured at two-minute intervals on the dominant arm, using an automated device (Dinamap).

After local anesthesia, a microdialysis probe (CMA 70 brain microdialysis catheter, Stockholm, Sweden) was inserted into the anterior forearm muscle compartment of the non-dominant arm, guided by a 14-gauge Venflon cannula. The probe had a dialysis tubing of 10x0.6 mm with a membrane cutoff of 20.000 Dalton. The probe was continuously perfused with 0.9% NaCl with a microdialysis pump (CMA 107, Stockholm, Sweden) at a rate of 2 µl/min. The effluent (dialysate) was collected at 15 minute intervals to obtain 30 µl samples, which were kept on ice and protected from light before they were stored at –20 °C until analysis.

In the main protocol (protocol 1), maximal force of handgrip was determined after insertion of the microdialysis probe (Baseline Hydrolic Hand Dynamometer, Fabrication Enterprise Inc., Irvington, NY, USA). Sampling of microdialysate started immediately after insertion of the microdialysis probe, when dialysate adenosine concentration is known to be particularly high, due to local muscle cell injury, returning to a stable baseline level within one hour (209). Therefore, baseline values were determined in 2 consecutive dialysate samples that were obtained 1 hour after insertion of the microdialysate probe. Thereafter, subjects performed intermittent handgrip during 15 minutes, with 5-second contractions at 50% of maximal force every 10-seconds while dialysate sampling continued. One hour later, this procedure was repeated during concomitant administration of dipyridamole into the brachial artery (12 µg/min/dl of forearm tissue) which

started 15 minutes before the second period of contractions and was continued throughout the exercise (n=9). In a time-control study, eight subjects performed two similar periods of exercise without administration of dipyridamole.

An additional series of experiments was performed in nine subjects to determine whether the endothelial barrier for adenosine is still intact after insertion of a microdialysis probe (protocol 2). In addition to the interstitial probe, a second, identical probe was inserted retrogradely into a deep antecubital vein of the same arm, guided by a 16-gauge Venflon cannula. One hour after instrumentation, baseline adenosine samples were obtained during intra-brachial infusion of normal saline. Subsequently, interstitial and circulating adenosine was measured during infusion of increasing dosages of adenosine (5, 15 and 50 μg/min/dl) into the brachial artery. Each dose was infused for 20 minutes followed by 5 minutes of wash-out. After one hour, again baseline values were obtained during infusion of normal saline followed by dipyridamole (100 μg/min/dl). Subsequently, the lowest dose of adenosine was repeated together with dipyridamole. In this protocol, we used a higher dose of dipyridamole compared with the first protocol, in order to prevent insufficient forearm concentrations of dipyridamole that could result from dilution as a result of adenosine-induced vasodilation. After completion of the second protocol, both microdialysis probes were removed and suspended in isotonic saline for *in vitro* calibration, as previously described (209). To determine the efficacy of delivery of dipyridamole into the interstitial compartment during intrabrachial administration, we measured dipyridamole concentration simultaneously with the intravascular and interstitial microdialysis probe in five subjects of protocol 2.

In the time-control group of protocol 1, we also determined the concentration of creatine and phosphocreatine in the dialysate samples. We presumed that an increase in (phospho)creatine would reflect local mechanical disrupture of cellular membranes of muscle cells in the vicinity of the microdialysis probe. To exclude the possibility that (phospho)creatine is also released by exercising viable muscle cells, an additional series of experiments was performed in eight healthy volunteers (four male, four female). After retrograde cannulation of the antecubital vein of the non-dominant arm, the volunteers performed 15 minutes of intermittent handgripping at 50% of maximal voluntary force, as described previously. Before and immediately after this exercise period, blood samples were taken for determination of plasma (phospho)creatine levels.

Analytical Procedures

Dialysate samples (15 μl) were analyzed for concentrations of adenosine using HPLC, equipped with a UV detector set at 254 nm. A binary low-pressure gradient elution was used with eluent A consisting of di-potassium-hydrogen-phosphate (0.1 M) and tetra-butyl-ammonium-hydrogen-sulfate (10 mM) as the ion-pair forming agent. The pH was adjusted to 6.5 with HCl. Solvent B contained, in addition, 40% (v/v) methanol. In addition, in the dialysate samples (10 μl) from the time-control study of protocol 1 the concentration of creatine was determined spectrophotometrically (kit nr. 12320, Merck) without use of creatininase. Also, for each sample, creatine was measured after enzymatic conversion of phosphocreatine by creatine kinase. The

concentration of phosphocreatine was calculated by taking the difference between these two creatine concentrations.
Dialysate dipyridamole concentration was determined using HPLC with fluorescence detection set at 286/470 nm.

Drugs and Solutions
Adenosine (*Adenocor*, Sanofi-Synthelabo) and dipyridamole (*Persantin*, Boehringer Ingelheim) were diluted in normal saline to obtain the different concentrations.

Data analysis and Statistics
In the first protocol, all blood pressure values during the 15-minute contraction periods were averaged to one value. This value was compared with the mean value of a baseline period of 15 minutes immediately before contraction. All results are expressed as mean ± SE, unless indicated otherwise.
The effect of dipyridamole on exercise-induced increases in adenosine and blood pressure was tested with a paired Student's t-tests. The effect of dipyridamole on interstitial and intravascular adenosine concentrations was assessed with repeated measures ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

Protocol 1: The effect of dipyridamole on muscle interstitial adenosine concentration and pressor response during exercise.
Infusion of dipyridamole into the brachial artery during the second period of contraction significantly potentiated the exercise-induced increase in dialysate adenosine from 0.30 ± 0.08 μmol/l during the first bout of exercise to 0.48 ± 0.10 μmol/l ($P<0.05$ for effect of dipyridamole). In contrast, dipyridamole did not potentiate the exercise-induced increase in blood pressure: 9.6 ± 2.4 / 4.5 ± 2.0 mmHg (SBP/DBP) and 10.4 ± 2.2 / 7.0 ± 1.3 in the absence and presence of dipyridamole, respectively ($P>0.1$).
In the time-control study, dialysate adenosine concentration increased by 0.25 ± 0.10 and 0.15 ± 0.07 μmol/l during the successive exercise periods ($P>0.1$ for the comparison between the first and the second period of contractions; n=8, figure 1). The exercise-induced rise in blood pressure (SBP/DBP) was 15.1 ± 2.8 / 9.8 ± 2 mmHg for the first contraction period and 17.3 ± 3.7 / 14.3 ± 1.6 for the second contraction period ($P>0.1$ for SBP, $P<0.05$ for DBP for comparison between first and second period).

In the time-control group, dialysate (phospho)creatine was high immediately after insertion due to mechanical injury of muscle cells (330 ± 82 μmol/l, n=7). After 90 minutes, (phospho)creatine had stabilized (65 ± 11 μmol/l). During subsequent exercise, dialysate (phospho)creatine increased by 151 ± 58 μmol/l. Before the second bout of exercise, (phospho)creatine had returned to baseline and increased by 45 ± 19 μmol/l during the second period of exercise. The ratio of dialysate (phospho)creatine to adenosine just after insertion did not significantly differ from the ratio during the exercise periods (1291 ± 477 versus 1033 ± 434 and 805 ± 181, $P>0.1$).

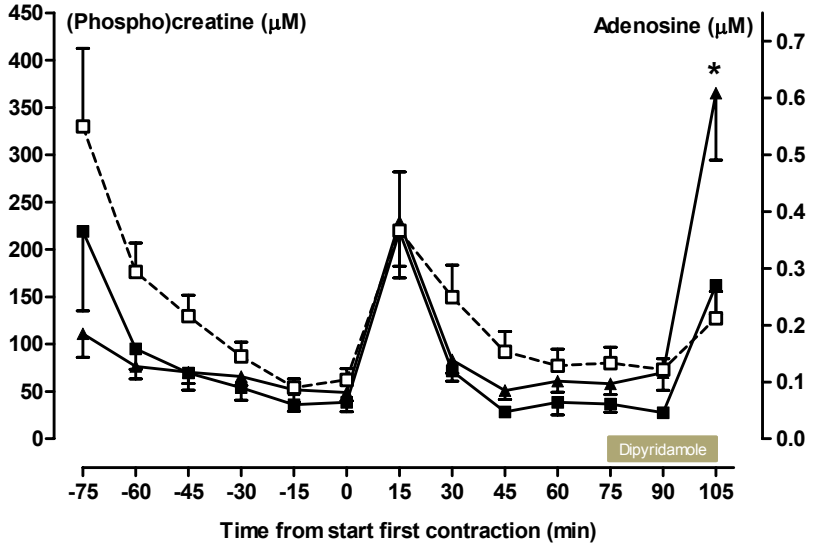


Figure 1: Dialysate concentrations of adenosine (filled squares, n=8) and (phospho)creatine (open squares, n=7) during two consecutive periods of intermittent isometric exercise. In a separate group of subjects, again dialysate adenosine was determined during a similar exercise protocol, but now dipyridamole was administered (grey bar) during the second exercise period (filled triangle, n=9). *significant potentiation of exercise-induced increase in adenosine by dipyridamole (P<0.05).

In an additional study, antecubital venous plasma (phospho)creatine concentrations were determined before and immediately after 15 minutes of rhythmic exercise. (Phospho)creatine concentration was 50 ± 28 μmol/l and 47 ± 22 μmol/l before and after exercise, respectively (n=8, P>0.1).

Protocol 2: Simultaneous measurement of interstitial and circulating adenosine during intrabrachial infusion of adenosine with and without dipyridamole

Intra-arterial infusion of only adenosine or dipyridamole did not significantly affect dialysate adenosine concentrations from either intravascular or intramuscular probe (figure 2).

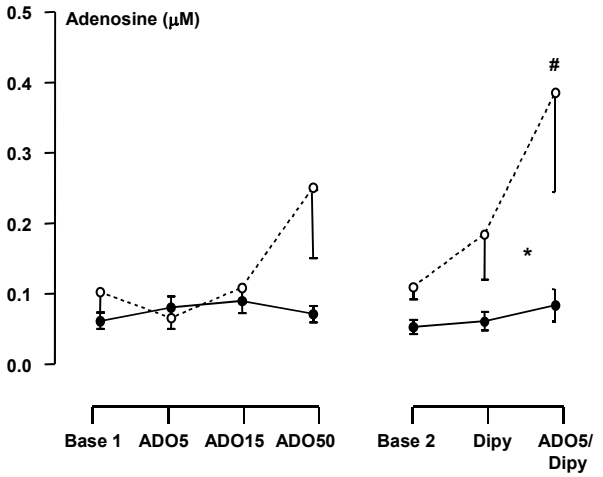


Figure 2: Simultaneously measured dialysate adenosine from the intramuscular probe (filled circles) and from the intravenous probe (open circles) during infusion of adenosine (left) and dipyridamole with and without adenosine (right) into the brachial artery. #Significant increase of circulating adenosine during the infusion of dipyridamole and adenosine (P<0.05). *Significant difference between the course in interstitial and circulating adenosine (P<0.05).

However, co-infusion of adenosine and dipyridamole increased adenosine in dialysate from the intravascular probe from 0.11 ± 0.02 during baseline to 0.39 ± 0.14 μmol/l (P<0.05; n=9), without affecting adenosine in the dialysate from the intramuscular probe (0.05 ± 0.01 μmol/l at baseline versus 0.08 ± 0.02 μmol/l during administration of dipyridamole and adenosine; P>0.1, n=9).
The *in vitro* adenosine recovery from the intramuscular and intravenous probes averaged 42 ± 4 versus 49 ± 6%, respectively (P>0.1).
During intrabrachial administration, dipyridamole effectively diffused into the interstitial compartment: the dialysate concentration of dipyridamole from the intravascular probe was 3.18 ± 1.6 μmol/l and from the interstitial probe 1.59 ± 1.0 μmol/l. Absolute concentration cannot be calculated because we did not determine recovery for dipyridamole.

DISCUSSION

In the present study, we showed that local infusion of the nucleoside uptake inhibitor dipyridamole into the brachial artery potentiates the handgrip exercise-induced increase in muscle interstitial adenosine but not the exercise-induced pressor response. This observation argues against a role for adenosine as a trigger for the exercise pressor reflex to intermittent static handgripping in healthy young volunteers.

It has long been known that exercise elicits sympathetic activation and a rise in blood pressure (615), which are induced both by central command and by activation of a reflex originating in the exercising skeletal muscle (616). The afferent limb of this reflex is composed of type III and IV

muscle afferents, which can be activated by mechanical as well as chemical stimuli (616). Much research has been devoted to elucidate the role of the endogenous nucleoside adenosine as one of these chemical triggers of the exercise pressor reflex, but still no consensus has been reached. Adenosine is an attractive candidate for triggering this reflex because it is known to excite a variety of afferent fibers. Activation of metabosensitive afferents by adenosine has been demonstrated in the kidney (630), in carotic chemoreceptors (81), forearm (79), and heart (78), although not all studies confirm this afferent fiber activation in the heart (631).
The concentration of endogenous adenosine in the muscle interstitial compartment, where the metabosensitive afferents that trigger the exercise pressor reflex are mainly located, can only be determined by microdialysis. In resting muscle, microdialysis provides an accurate method to determine interstitial adenosine (209, 626). However, using microdialysis to measure muscle interstitial adenosine during muscle contraction is more complicated. It has been suggested that in this situation the microdialysis probe injures muscle cells in the vicinity of the probe (629). Consequently, an exercise-induced increase in dialysate adenosine might not reflect a genuine increase in interstitial adenosine but rather local mechanical cellular disrupture. To circumvent this possible source of confounding, we used dipyridamole as pharmacological tool to inhibit adenosine uptake during exercise, to further increase the extracellular interstitial adenosine concentration without a concomitant increase in mechanical damage. We showed that infusion of dipyridamole into the brachial artery significantly potentiates the exercise-induced increase in dialysate adenosine, whereas dipyridamole does not potentiate the pressor response.

On the basis of these observations, we concluded that dipyridamole effectively inhibits adenosine uptake in the interstitial compartment. This conclusion is not confounded by any local mechanical cellular injury because the source of the increased adenosine is not relevant. However, this conclusion could critically be confounded by disruption of the endothelial barrier for adenosine by either dipyridamole (53) or mechanically by insertion of the microdialysis probe. In that case, extravasation of increased circulating adenosine could account for the observed increase in dialysate adenosine. Therefore, in an additional series of experiments, we tested whether the endothelial barrier for adenosine is still intact during interstitial microdialysis. We simultaneously measured interstitial adenosine with a microdialysis probe inserted into the flexor muscle of the forearm, and circulating adenosine with a microdialysis probe inserted retrogradely into the medial cubital vein during concomitant infusion of dipyridamole and adenosine into the brachial artery. Infusion of adenosine and dipyridamole significantly increased dialysate adenosine from the intravascular probe but not from the interstitial probe. This clearly confirms that after insertion of a microdialysis probe in forearm muscle, the endothelial barrier for adenosine is still intact. Therefore, the potentiation of the exercise-induced increase in dialysate adenosine by dipyridamole indeed represents a genuine increase of adenosine in the interstitial compartment.

Up to now, three types of experiments have been performed to study the role of adenosine in triggering the pressor reflex and each has yielded inconsistent results. First, exogenous adenosine has been injected locally into the arterial supply of limbs. Indeed, injection of adenosine into the brachial artery in humans increases blood pressure (79, 617-619). Moreover, this is not caused by venous spillover of adenosine, because adenosine was administered during venous occlusion (79,

617-619), because intravenous administration of the same dose did not affect blood pressure (618), and because the blood pressure response was blunted by axillary ganglionic blockade (617). In contrast, in anesthetized cats and rabbits, intra-arterial injection of adenosine or the stable adenosine analogue 2-chloroadenosine did not elicit pressor responses (620, 621, 625). In particular, Maclean *et al* administered adenosine into the arterial supply of cat triceps surae muscle *in vivo* in a sufficient dose to significantly increase muscle interstitial adenosine, but they still did not observe any hemodynamic changes (621). This discrepancy is attributed to the effects of anesthesia and substantial interspecies differences in the actions of adenosine (128). Although the evidence that exogenous adenosine stimulates muscle afferents in the forearm is very robust (79, 617, 618), no conclusion can be derived considering the role of endogenous adenosine during exercise. The endothelium acts as a very strong metabolic barrier for adenosine between the intravascular and interstitial compartment (52). In the cat hindlimb, < 3% of the intraluminally administered adenosine dose diffused into the interstitial compartment, where the metabosensitive afferent fibers reside (621). Consequently, intravenously administered exogenous adenosine probably does not directly stimulate the metabosensitive afferents, which are involved in the pressor response to exercise.

Second, the role of adenosine as trigger of the pressor reflex has been studied by the use of adenosine receptor antagonists during exercise. Costa *et al* demonstrated that intra-arterially administrated theophylline attenuates the pressor response to sustained isometric handgrip (79), whereas Notarius *et al* showed that systemic infusion of caffeine does not affect the pressor response to isometric handgrip and posthandgrip ischemia in healthy subjects but does so only in patients with cardiac failure during posthandgrip ischemia (624).

Third, studies directly measuring endogenous interstitial adenosine have contributed to increasing knowledge on the role of adenosine in the exercise pressor reflex. It has long been impossible to actually measure endogenous adenosine in vicinity of the muscle afferents in the interstitium, because of the very short half-life of adenosine (51). Moreover, circulating adenosine does not reflect interstitial adenosine because of the strong endothelial barrier for adenosine (52). With microdialysis, direct measurement of interstitial adenosine is possible. Using microdialysis probes inserted into exercising muscle, several groups have demonstrated that dialysate adenosine increases during exercise (626-628). Moreover, a role for adenosine as trigger in the pressor reflex was suggested by showing that the dialysate adenosine concentration correlates to exercise intensity on the one hand and to the increase in MSNA on the other hand (617).

Recently, Nordsborg *et al* provided evidence that, when using microdialysis in exercising muscle, local mechanical disrupture of cellular membranes of muscle cells in the vicinity of the probe occurs (629). They showed that during exercise, high concentrations of carnosine were found in the dialysate. As carnosine is found in high concentrations in muscle cells but not in plasma, this finding indicates disrupture of cellular membranes. Because the intracellular and extracellular concentration of purine nucleotides and nucleosides also importantly differ, membrane rupture might significantly confound measurements of interstitial adenosine. To determine whether local muscle cell injury also occurred in our experiments, we measured the dialysate concentrations of (phospho)creatine. (Phospho)creatine is found in high intracellular concentrations in muscle cells, but it cannot pass the cellular membrane (632). We assumed that an increase of these substances in

the dialysate implicates local mechanical disrupture of the membranes of cells located in the vicinity of the microdialysis probe. Immediately after insertion of the microdialysis probe, dialysate levels of adenosine and (phospho)creatine are elevated, reflecting pure mechanical injury of muscle fibers (figure 1). During exercise, a similar increase in (phospho)creatine and adenosine occured and the ratio of (phospho)creatine to adenosine did not significantly differ between samples obtained during exercise and those obtained immediately after insertion of the probe, although there was a trend toward a reduction of this ratio. This observation suggests that local mechanical injury of muscle cells significantly contributes to the increase of adenosine in the dialysate during exercise. To exclude that exercising muscle cells release creatine without mechanical damage, we measured venous (phospho)creatine in a separate group of subjects before and immediately after 15 minutes of intermittent isometric forearm exercise. This exercise protocol was not accompanied by an increase in forearm venous (phospho)creatine, which suggests that exercise itself does not trigger the release of this substance by viable cells.
In general, our findings suggest that microdialysis is not well suited for measurement of interstitial purines in exercising muscle, which is very important in the interpretation of previously published studies on this topic.

On the basis of the abovementioned observations, we conclude that adenosine does not importantly trigger the pressor response to intermittent handgrip in these healthy young volunteers. It is important to realize that the isometric exercise used in the present study, as in previous studies with microdialysis, was performed rhythmically without superimposed ischemia to allow volunteers to sustain the exercise for a sufficient period of time to complete microdialysis sampling. Therefore, skeletal muscle perfusion was restored during each 5 second interval of relaxation, thus potentially allowing wash out of accumulated metabolites. In contrast, in the study by Costa and Biaggioni, in which it was demonstrated that theophylline inhibits the exercise-induced sympathetic activation and pressor response, sustained isometric handgrip was used (79). The intermittent restoration of muscle perfusion in our study prevents accumulation of metabolites such as adenosine and could explain why in our experimental set up, adenosine is not importantly involved in triggering the pressor reflex. This phenomenon could also well explain the previous observation that the adenosine receptor antagonist caffeine inhibits the pressor response to exercise in patients with heart failure, who have compromised skeletal muscle perfusion, but not in healthy controls (624). The intermittent non ischemic exercise performed in the present study is highly relevant to daily life. However, using this particular stimulus, the observed pressor response is resulting from stimulation of chemo- and mechanosensitive afferents as well as from central command. Nevertheless, if adenosine were involved as one of the stimuli of metabosensitive afferents, a significant increase in interstitial adenosine resulting from dipyridamole infusion should have potentiated the pressor response, even when central command and mechanoreceptor stimulation also contribute to sympathetic activation.
As caffeine is a potent adenosine receptor antagonist, it is important to consider the duration of caffeine abstinence before experimentation. In the present study, subjects abstained from caffeine-containing beverages for at least 24 hours, whereas in some previous studies on this topic, 72 hours of abstinence was used (79). Rongen *et al* studied the effect of the duration of caffeine abstinence (6, 30, 78, 150 and 318 hours) on the hemodynamic effects of acute systemic administration of

adenosine (118). They showed that this duration did not influence heart rate and diastolic blood pressure responses. The adenosine-induced increase in systolic blood pressure was significantly higher only after one week of abstinence. Consequently, studies using caffeine abstinence for one or three days can be compared without confouding by this variable.
Finally, it needs to be realized that dipyridamole is proposed to have alternative mechanisms of actions besides inhibition of nucleoside transport (103). However, our group has shown previously that dipyridamole-induced vasodilation in the concentration range used in the present study is indeed due to adenosine uptake inhibition, because it could be inhibited by the adenosine receptor antagonist theophylline (106).

In conclusion, in the present study, we showed that dipyridamole effectively inhibits interstitial adenosine uptake, but does not potentiate the pressor response to intermittent isometric handgrip, which argues against a role for adenosine as a metabolic trigger of the exercise pressor response.

ACKNOWLEDGEMENTS
NP Riksen is a MD-clinical research trainee financially supported by the Netherlands Organization for Scientific Research (ZonMw). The contribution of GA Rongen has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences (KNAW). The contribution of PHH van den Broek has been made possible by European Union support (project number QLK1-CT-2000-00069).

CHAPTER 5

SUMMARY AND CONCLUSIONS

In the last decades increasing knowledge about the metabolism of adenosine and the effects of adenosine receptor stimulation has led to the unifying concept of adenosine as "retaliatory metabolite" (16, 71): in situations of impending tissue danger, such as ischemia, changes in formation, transport, and degradation of adenosine result in a rapid increase in the local extracellular adenosine concentration. Subsequent stimulation of adenosine receptors induces various effects, such as vasodilation, preconditioning, modulation of sympathetic nervous system activity, inhibition of inflammation, and modulation of vascular cell proliferation and death, aimed at protecting the affected tissue against the insult.

During ischemia and reperfusion, these effects have the potential to potently limit myocardial infarct size (203, 633). In addition, several studies suggest that in the long run adenosine receptor stimulation inhibits the development of atherosclerotic lesions and promotes angiogenesis (17, 19, 20). Therefore, variations in the metabolism of adenosine and in stimulation of adenosine receptors could well affect individual cardiovascular risk. Indeed, it was shown previously that the 34C>T variant of the *AMPD1* gene, predicting increased adenosine formation during ischemia, is associated with reduced cardiovascular mortality in patients with coronary artery disease (21). In the present thesis, we aimed to identify genetic, metabolic, and pharmacological determinants of the cardiovascular effects of adenosine, which could potentially affect individual cardiovascular risk. In the various human *in vivo* studies, which are described in this thesis, two end-points of adenosine receptor stimulation are used: vasodilation, assessed with venous occlusion plethysmography in the forearm, and ischemia-reperfusion injury as determined with ^{99m}Tc-annexin A5 scintigraphy of the thenar muscle. The latter method is based on the high-affinity binding of annexin A5 to phosphatidylserine residues, which are exposed on the outside of cellular membranes early after an ischemic insult, as early marker of ischemia-reperfusion injury (134). The results of these experiments are summarized in figure 1.

In chapter 2, we explored the effects of two common polymorphisms in the genes encoding for adenosine monophosphate deaminase (*AMPD1*) and the adenosine A_{2A} receptor (*ADORA2A*) on the vascular effects of adenosine. The 34C>T variant of *AMPD1* encodes a truncated protein with loss of function (138). We hypothesized that in subjects with this variant allele, during ischemia intracellular conversion of AMP into adenosine is augmented, which decreases the transmembranous adenosine concentration gradient and reduces inward facilitated diffusion of extracellular adenosine, resulting in increased adenosine receptor stimulation. Indeed, in **chapter 2.1** we demonstrated that in healthy volunteers heterozygous for the 34C>T variant post-occlusive reactive hyperemia is augmented as compared to subjects homozygous for the common allele. The observation that the potentiating effect of the ENT inhibitor dipyridamole on reactive hyperemia is reduced in these subjects is compatible with augmented intracellular adenosine formation and a reduced transmembranous adenosine concentration gradient. Also, we demonstrated that in subjects with the CT genotype ischemia-reperfusion injury in the thenar muscle, as assessed with ^{99m}Tc-annexin A5 scintigraphy, is reduced. These mechanisms could well contribute to the survival benefit of cardiovascular patients with this variant allele (21, 119).

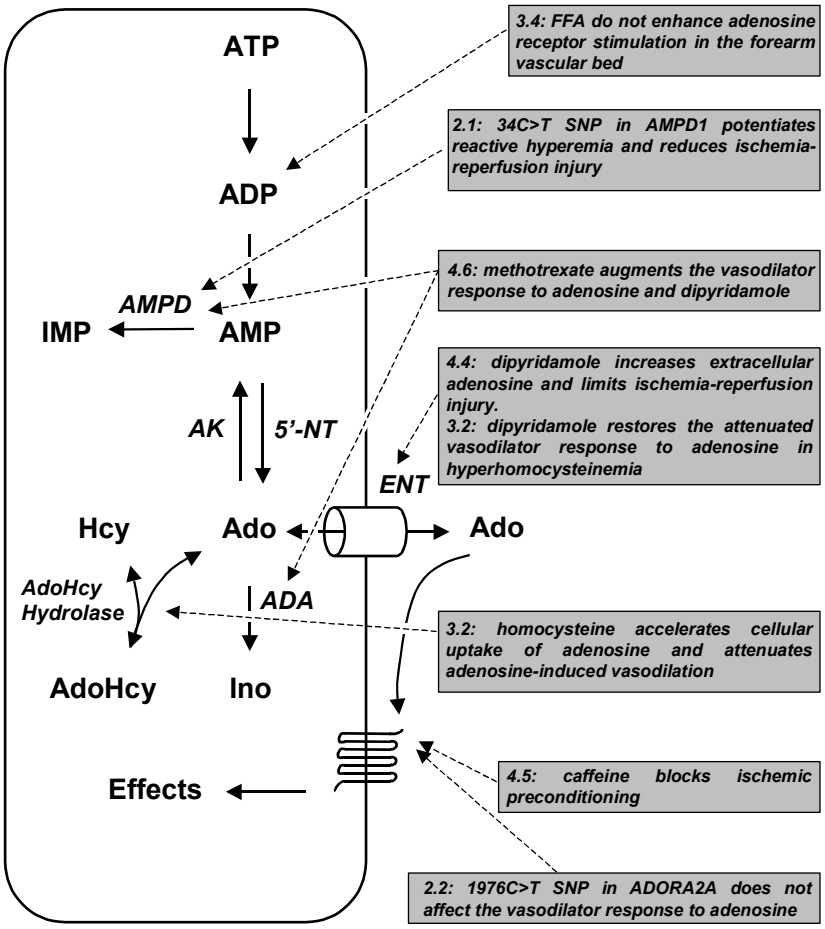


Figure 1: schematic overview of the genetic, metabolic, and pharmacological determinants of adenosine receptor stimulation. The dashed arrows indicate the proteins which are influenced by these factors. The numbers indicate the chapters in which the results are described.

In **chapter 2.2** we explored the effect of the 1976C>T variant in *ADORA2A* on the vasodilator action of adenosine. This particular polymorphism was selected because previous studies suggested that homozygosity for the variant allele could affect the function of the adenosine A_{2A} receptor (120). We demonstrated that the forearm vasodilator response to the administration of adenosine into the brachial artery was not different between healthy volunteers with the CC genotype and the TT genotype. Therefore, it is unlikely that this polymorphism could potentially affect individual cardiovascular risk.

In chapter 3 we explored whether the metabolism of adenosine and subsequent adenosine receptor stimulation could be affected by metabolic compounds such as homocysteine and free fatty acids

(FFA) and, as such, could play a role in the adverse cardiovascular sequelae of diseases in which plasma concentrations of these compounds are elevated. In **chapter 3.1** we postulate that in patients with hyperhomocysteinemia increased intracellular formation of S-adenosylhomocysteine reduces free intracellular adenosine. Subsequently, facilitated diffusion of extracellular adenosine into cells through the ENT transporter is enhanced, thus limiting adenosine receptor stimulation. In **chapter 3.2** we tested this hypothesis and we demonstrated that in isolated erythrocytes homocysteine indeed accelerates uptake of extracellular adenosine, which is associated with increased intracellular formation of S-adenosylhomocysteine, and which can be inhibited by dipyridamole. Subsequently, we showed that in patients with severe hyperhomocysteinemia due to cystathionine beta-synthase deficiency, adenosine-induced forearm vasodilation is reduced and that this can be completely restored by the concomitant administration of dipyridamole. These effects of homocysteine on adenosine kinetics, resulting in reduced adenosine receptor stimulation, could well contribute to the increased cardiovascular risk of patients with hyperhomocysteinemia. The evidence in favor of this mechanism is reviewed in **chapter 3.3**. In **chapter 3.4** we tested the hypothesis that FFA increase the extracellular adenosine concentration by inhibition of the mitochondrial adenine nucleotide translocator (121). By this mechanism, adenosine could be involved in the hyperdynamic circulation in patients with the metabolic syndrome, which is typically associated with elevated plasma FFA. We showed in healthy subjects that acute elevation of plasma FFA induced forearm vasodilation. However, this was not due to increased adenosine receptor stimulation because caffeine did not antagonize this vasodilator response. These observations suggest that adenosine is not involved in the hemodynamic consequences of elevated plasma free fatty acids, such as in patients with the metabolic syndrome.

Chapter 4 is dedicated to pharmacological modulation of the metabolism and receptor binding of adenosine. We were mainly interested in the effects of these pharmacological interventions on ischemia-reperfusion injury. Unfortunately, easy-to-use models to study ischemia-reperfusion injury in humans *in vivo* were lacking. Therefore, we developed ^{99m}Tc-annexin A5 scintigraphy, which is based on the high-affinity binding of annexin A5 to phosphatidylserine residues, which are exposed on the outer membrane leaflet of cells early after an ischemic insult. By labeling recombinant annexin A5 to ^{99m}Technetium, it is possible to detect this early marker of ischemia-reperfusion injury with a gamma camera. In **chapter 4.3** we describe that 10 minutes of ischemic handgripping of the non-dominant arm induces a significant targeting of annexin A5 to the thenar muscle of the experimental hand as compared to the control hand. Subsequently, we showed that 10 minutes of forearm ischemia prior to the 10 minutes of ischemic exercise, as a stimulus for ischemic preconditioning, significantly reduced annexin targeting. In **chapters 4.1 and 4.2** we describe in detail the phenomenon of ischemic preconditioning and its potential modulation by drugs. In addition, administration of adenosine into the brachial artery for 10 minutes prior to ischemic exercise also resulted in a potent reduction in annexin targeting. In conclusion, we demonstrated that ^{99m}Tc-annexin A5 scintigraphy provides a reliable human *in vivo* model to detect ischemia-reperfusion injury and modulation of this injury by ischemic or pharmacological preconditioning. In clinical practice, the use of ischemic preconditioning to increase tolerance against ischemia-reperfusion injury is limited because the stimulus for preconditioning has to be applied shortly before the actual ischemic insult, which is mostly not predictable. In **chapter 4.4** we

used annexin A5 scintigraphy to study whether chronic oral treatment with dipyridamole could offer more prolonged protection against ischemia-reperfusion. In healthy volunteers we showed that the use of dipyridamole (dipyridamole retard, 200 mg bid, for one week) significantly reduced annexin A5 targeting in the thenar muscle after 10 minutes of ischemic exercise as compared to control subjects. Because it is well-known that endogenous adenosine is a key mediator in the protective effect of ischemic preconditioning, our next hypothesis was that the adenosine receptor antagonist caffeine limits this protective effect. In **chapter 4.5** we addressed this hypothesis with the ^{99m}Tc-annexin A5 model. In a randomized double-blinded trial in healthy subjects, we showed that pretreatment with caffeine, in a final plasma concentration that is reached in daily life by drinking 2-4 cups of regular coffee, completely abolished the protective effect of ischemic preconditioning. In order to study whether these observations in skeletal muscle could be extrapolated to the heart, we used post-ischemic recovery of contractile function of human atrial trabeculae, harvested during heart surgery, as an alternative model to study ischemia-reperfusion injury. Also in this model, caffeine completely abolished the protective effect of ischemic preconditioning on recovery of contractile function after simulated ischemia and reperfusion.

In additional to direct effects on the cardiovascular system, adenosine also acts as a potent immunomodulator: during inflammation, the concentration of adenosine increases and subsequent adenosine receptor stimulation of immune cells attenuates the immune response (10). Therefore, drugs that increase the adenosine concentration could be expected to have anti-inflammatory effects. Indeed, it has been shown in animal models that the anti-inflammatory effects of methotrexate are mediated by increased adenosine receptor stimulation. It has been suggested that by indirect inhibition of adenosine deaminase and AMPD, methotrexate decreases intracellular degradation and increases extracellular formation of adenosine (584). In **chapter 4.6** we aimed to confirm this effect of methotrexate on the metabolism of adenosine in humans *in vivo*. We showed that treatment with methotrexate for 12 weeks (15 mg per week orally in patients with arthritis) augments the forearm vasodilator response to the administration of adenosine into the brachial artery. This finding is compatible with impaired intracellular degradation of adenosine. In addition, dipyridamole-induced forearm vasodilation was also potentiated by methotrexate, which is compatible with increased extracellular formation of adenosine. Given the various cardiovascular effects of adenosine, we hypothesized that methotrexate also has potential beneficial cardiovascular effects. Indeed, in a large epidemiological trial, methotrexate reduced cardiovascular mortality in patients with rheumatoid arthritis (607), although another study could not confirm this finding (608). Interestingly, a recent study in patients with heart failure showed that treatment with methotrexate improved NYHA functional class and 6-minute walk test distance (612). In **chapter 4.7** we discuss the possibility that these effects could be due to increased adenosine receptor stimulation.

Finally, in **chapter 4.8** we used dipyridamole as a pharmacological tool to inhibit the ENT transporter in order to explore the role of endogenous adenosine as a trigger of the exercise pressor reflex. The exercise pressor reflex is defined as the rise in arterial blood pressure that results from strenuous exercise of skeletal muscle. Despite much previous effort to test the hypothesis that this reflex is mediated by reflex activation of sympathetic afferents in the exercising muscle by

adenosine, released during muscle contraction, this mechanism remains controversial. In our study, healthy volunteers performed rhythmic forearm exercise to activate the pressor response. The muscle interstitial adenosine concentration was measured with microdialysis. We showed that the intrabrachial administration of dipyridamole during forearm exercise potentiates the exercise-induced increase in muscle interstitial adenosine, but does not potentiate the pressor response. Therefore, these observations provide evidence against a role for adenosine as trigger of the exercise pressor reflex.

CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

The cardiovascular effects of adenosine are of major importance, as they have the potential to reduce myocardial infarct size and prevent the development of atherosclerosis. Therefore, drugs that activate adenosine receptors, either directly or by increasing the endogenous adenosine concentration, could be of potential benefit for patients suffering from or at risk for cardiovascular disease. Although animal studies about the beneficial effects of adenosine on cardiovascular end-points are abundant, human *in vivo* data are scarce. In two studies in patients with acute myocardial infarction undergoing reperfusion therapy, it was shown that intravenous infusion of adenosine for three hours reduced infarct size as assessed with ^{99m}Tc sestamibi tomography, although clinical outcome was not significantly improved (511, 634). The use of adenosine in clinical practice in patients prone for cardiovascular events is limited by its extremely short half life, necessitating continuous intravenous infusion, and by systemic side effects. The use of selective adenosine subtype receptor agonists could potentially overcome these problems. Several agonists of adenosine receptor subtypes have already entered phase III clinical trials, but not (yet) for the purpose of cardiovascular protection (65). For example, selective adenosine A_1 receptor agonists have been tested for its capacity to terminate paroxysmal supraventricular tachycardias (635), and selective adenosine A_{2A} receptor agonists have been evaluated for myocardial stress testing (636).

An alternative, and conceptually more appealing approach to exploit the cardiovascular protective effects of adenosine receptor stimulation in clinical practice, would be the use of allosteric enhancers of adenosine receptors, because these compounds allow the effects of endogenous adenosine to be selectively magnified in a time- and site-specific manner (637). Allosteric enhancers of adenosine receptors enhance responsiveness of the receptor to its endogenous ligand adenosine and, as such, the effect of allosteric enhancers is limited to sites and times of adenosine accumulation, such as during ischemia. Phase I trials with an allosteric adenosine A_1 receptor enhancer have already been performed and phase II trials are planned (T-62, King's Pharmaceuticals).

Event-responsive and temporally specific potentiation of adenosine receptor stimulation can also be achieved with drugs that interfere with the metabolism and transport of adenosine, such as dipyridamole. Dipyridamole increases the endogenous adenosine concentration, especially in situations of increased adenosine formation, such as during ischemia, and has the advantage that it can be administered orally, is safe and has few side-effects (558). Indeed, we demonstrated that oral administration of dipyridamole limits ischemia-reperfusion injury in forearm skeletal muscle as assessed with annexin A5 scintigraphy. This finding is compatible with the results of the recently

published ESPRIT trial in patients with a recent cerebrovascular event (638). This trial demonstrated that the combination of dipyridamole and acetylsalicylic acid is more effective than acetylsalicylic acid alone in the secondary prevention of vascular death, stroke, or myocardial infarction.

Acadesine (5-aminoimidazole-4-carboxamide riboside) is yet another drug that interferes with the metabolism of adenosine and could potentially increase the interstitial adenosine concentration during ischemia. In a recent study in patients undergoing coronary artery bypass grafting, acadesine (intravenous infusion for 7 hours, starting 15 minutes before anesthesia) was tested for its effect to limit ischemia-reperfusion injury. Acadesine did not reduce the incidence of myocardial infarction, but in patients who developed an acute myocardial infarction, acadesine significantly decreased mortality, which is compatible with pharmacological preconditioning (639).

Unfortunately, the most widely consumed drug worldwide, caffeine, is an adenosine receptor antagonist and, as such, could well have adverse cardiovascular effects. We demonstrated that caffeine, in a concentration reached after regular coffee consumption, completely abolished the protective effects of ischemic preconditioning. In epidemiological trials, the association between coffee consumption and the incidence of myocardial infarction is still controversial, although a recent trial demonstrated that intake of coffee was associated with an increased risk of myocardial infarction, but only among individuals with slow caffeine metabolism, suggesting that caffeine does play a role in this association (579). However, considering the protective effects of ischemic preconditioning, caffeine is not expected to increase the incidence of myocardial infarction *per se*, but mainly to adversely affect outcome after myocardial infarction in patients with angina pectoris. Also, the beneficial effects of dipyridamole would be expected to be reduced in patients with regular coffee consumption.

Not only the cardiovascular effects of adenosine can be exploited in clinical practice, but also its potent anti-inflammatory effects, and its effects in the nervous system, the kidney, and the lung, which is recently reviewed by Jacobson and Gua (65). Methotrexate has been shown to increase the extracellular adenosine concentration, and in animal models the anti-inflammatory effects of methotrexate are completely abolished by adenosine receptor antagonists. Also in humans it has been suggested that the efficacy of methotrexate in patients with rheumatoid arthritis is lower in patients with a high coffee intake, although a recent prospective cohort study did not confirm this association (640). We postulate that also other drugs that are known to increase the extracellular adenosine concentration, such as dipyridamole or acadesine, would be effective in reducing inflammatory activity in patients with rheumatoid arthritis.

Drugs that are aimed to facilitate adenosine receptor stimulation could be used in patients who are at increased risk for cardiovascular events to limit the extent, and perhaps even the incidence, of ischemic events. However, based on the results of the studies described in this thesis, it is to be expected that specific subgroups of patients would benefit even more from this pharmacological approach. For example, we showed that in patients with severe hyperhomocysteinemia, the vascular effects of adenosine are limited by accelerated cellular uptake and degradation of

extracellular adenosine, and that this could be completely restored by dipyridamole. Therefore, we hypothesize that dipyridamole could well reduce cardiovascular mortality in these patients.

Finally, polymorphisms or mutations in genes encoding for proteins involved in the metabolism or receptor binding of adenosine could potentially determine the efficacy of drugs that are aimed to magnify adenosine receptor stimulation. In this thesis, we have demonstrated that the effect of dipyridamole on post-occlusive reactive hyperemia is determined by the *AMPD1* genotype. We postulate that the beneficial cardiovascular effects of dipyridamole are reduced in patients with the 34C>T variant of the *AMPD1* gene because the transmembranous adenosine concentration gradient, which is the driving force for facilitated diffusion of adenosine, is less in these patients. In addition to determining the effects of pharmacological interventions, genetic variation can be used in the future to stratify cardiovascular risk in individual patients. We hypothesize that, in addition to the 34C>T polymorphism in the *AMPD1* gene, genetic variation in other proteins, such as adenosine deaminase, adenosine kinase, the ENT transporter, and specific adenosine receptors, could also affect individual cardiovascular risk.

In conclusion, the genotypes of proteins involved in the metabolism and receptor binding of adenosine could be used to predict cardiovascular risk in individual patients. In addition, based on animal studies, proof-of-concept studies in healthy volunteers, and some epidemiological evidence in patient studies, drugs that are aimed to modulate adenosine receptor stimulation have the potential to confer protection against cardiovascular diseases. To optimize the use of these drugs in clinical practice, more basic research is needed to develop novel drugs to potentiate adenosine receptor stimulation, more translational research is needed to study the effects of these compounds in humans *in vivo*, and more epidemiological studies are needed to explore the effects of these drugs on cardiovascular outcome in patients at risk for cardiovascular disease.

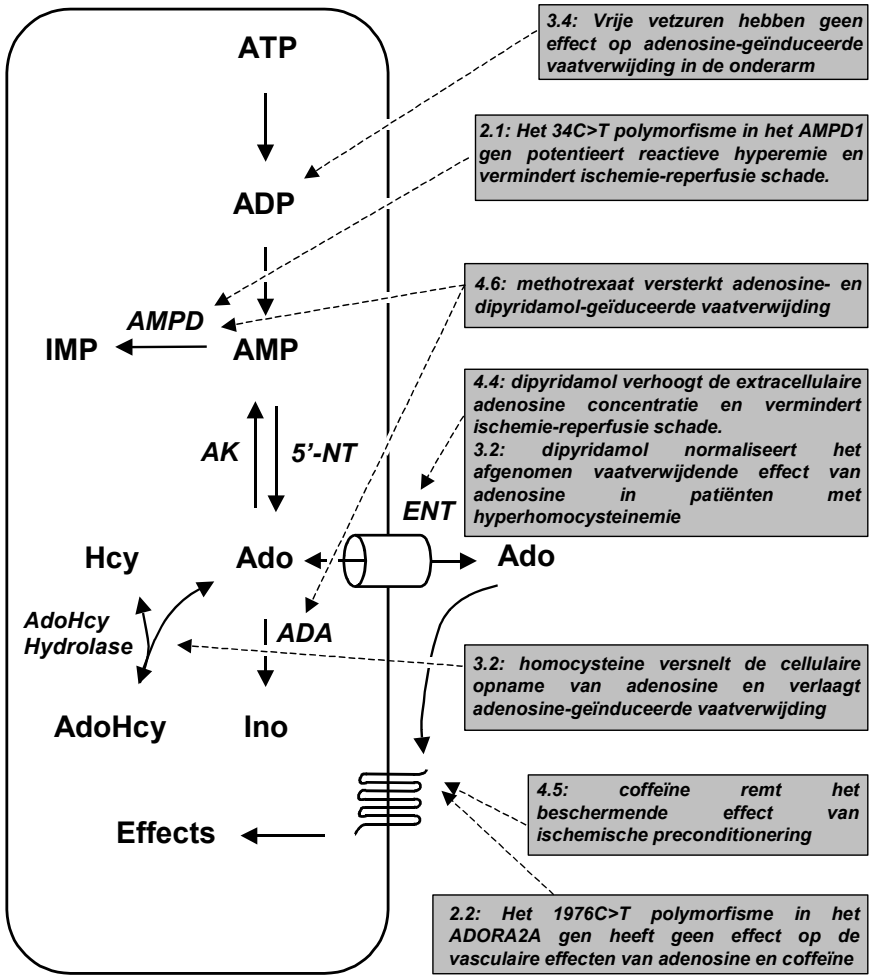
CHAPTER 6

NEDERLANDSE SAMENVATTING EN CONCLUSIES

In de afgelopen decennia is de kennis over het metabolisme van adenosine en de effecten van adenosinereceptorstimulatie sterk toegenomen. Dit heeft uiteindelijk geleid tot het concept van adenosine als "retaliatory metabolite" (16, 71): in situaties waarin weefselschade dreigt, zoals tijdens ischemie, treden er veranderingen op in de aanmaak, het transport en de afbraak van adenosine, waardoor lokaal de extracellulaire adenosine concentratie snel stijgt. Vervolgens leidt toegenomen stimulatie van adenosinereceptoren tot verschillende effecten, die het bedreigde weefsel kunnen beschermen, zoals vaatverwijding, "preconditionering", modulatie van de activiteit van het sympathische zenuwstelsel, ontstekingsremming en regulatie van de proliferatie en celdood van cellen in de vaatwand.

Tijdens ischemie en reperfusie kunnen deze effecten een forse reductie van de grootte van een myocardinfarct bewerkstelligen (203, 633). Daarnaast hebben verschillende studies het aannemelijk gemaakt dat adenosinereceptorstimulatie op de lange termijn het ontstaan van atherosclerose kan tegengaan en angiogenese kan bevorderen (17, 19, 20). Daarom zou interindividuele variatie in het metabolisme van adenosine en de receptorstimulatie door adenosine mogelijk van invloed kunnen zijn op het individuele cardiovasculaire risico. Inderdaad werd recent aangetoond dat in patiënten met coronaire hartziekten de 34C>T variant van het *AMPD1* gen geassocieerd is met een lagere cardiovasculaire mortaliteit, meest waarschijnlijk doordat deze variant leidt tot een sterkere stijging van adenosine tijdens ischemie (21). Het doel van dit proefschrift was het identificeren van genetische, metabole en farmacologische determinanten van de cardiovasculaire effecten van adenosine, die mogelijk het individuele cardiovasculaire risico zouden kunnen beïnvloeden. In de verschillende humane *in vivo* studies die in dit proefschrift zijn beschreven, gebruiken wij twee eindpunten van adenosinereceptorstimulatie: vaatverwijding, gemeten met veneuze occlusie plethysmografie in de onderarm, en ischemie-reperfusieschade, gemeten met ^{99m}Tc-annexine A5 scintigrafie van de duimmuisspier. Deze laatste methode is gebaseerd op het feit dat annexine A5 met een hoge affiniteit bindt aan fosfatidylserinegroepen. Deze moleculen verschijnen kort na het optreden van ischemie aan de buitenzijde van de celmembraan, als vroege *marker* van ischemie-reperfusieschade (134). Figuur 1 geeft een schematisch overzicht van de resultaten van deze experimenten.

In hoofdstuk 2 onderzochten wij het effect van twee veel voorkomende polymorfismen in de genen die coderen voor het adenosinemonofosfaat deaminase (*AMPD1*) en voor de adenosine A_{2A} receptor (*ADORA2A*) op de vasculaire effecten van adenosine. De 34C>T variant van het *AMPD1* gen introduceert een prematuur stopcodon en leidt tot een dysfunctioneel eiwit (138). Wij veronderstelden dat dit polymorfisme de intracellulaire omzetting van AMP in adenosine tijdens ischemie versterkt, waardoor de concentratiegradiënt van adenosine over de celmembraan afneemt en er dus minder gefaciliteerde diffusie is van extracellulair adenosine de cel in. Dit resulteert uiteindelijk in een toegenomen adenosinereceptorstimulatie.



Figuur 1: Schematisch overzicht van de genetische, metabole en farmacologische determinanten van adenosinereceptorstimulatie. De gestippelde pijlen duiden de eiwitten aan die door deze factoren worden beïnvloed. De getallen verwijzen naar de hoofdstukken in dit proefschrift waarin de desbetreffende resultaten zijn beschreven.

In **hoofdstuk 2.1** toonden wij in een studie bij gezonde vrijwilligers aan dat de reactieve hyperemie die optreedt na een periode van ischemie sterker is in proefpersonen die heterozygoot zijn voor de 34C>T variant dan in proefpersonen zonder deze variant. In de heterozygote proefpersonen was tevens het versterkende effect van de ENT remmer dipyridamol op de reactieve hyperemie verminderd. Dit duidt inderdaad op een versterkte intracellulaire vorming van adenosine tijdens ischemie en een afgenomen concentratiegradiënt van adenosine over de celmembraan. In deze zelfde studie lieten we zien dat ischemie-reperfusieschade na een periode van ischemie van de onderarm, zoals gemeten met ^{99m}Tc-annexine A5 scintigrafie, minder is in proefpersonen met het

CT genotype. Deze mechanismen zouden goed kunnen bijdragen aan het overlevingsvoordeel van patiënten met hart- en vaatziekten die dit polymorfisme hebben (21, 119). In **hoofdstuk 2.2** onderzochten wij het effect van de 1976C>T variant van het *ADORA2A* gen op het vaatverwijdende effect van adenosine. We onderzochten dit specifieke polymorfisme omdat eerder was aangetoond dat proefpersonen die homozygoot zijn voor deze variant een sterkere angstrespons hebben op het toedienen van de adenosinereceptorantagonist coffeïne, hetgeen suggereert dat dit polymorfisme de functie van de A_{2A} receptor kan veranderen (120). Het vaatverwijdende effect van adenosine in de onderarm en het effect van het toevoegen van coffeïne bleek niet te verschillen tussen gezonde vrijwilligers met het CC genotype en het TT genotype. Deze observatie maakt het onwaarschijnlijk dat dit polymorfisme een effect heeft op het individuele cardiovasculaire risico.

In hoofdstuk 3 onderzochten we of het metabolisme van adenosine en de vasculaire effecten van adenosine beïnvloed kunnen worden door homocysteine en vrije vetzuren en of adenosine dus een rol zou kunnen spelen in het verhoogde cardiovasculaire risico van patiënten met stofwisselingsziekten waarbij de concentraties van homocysteine of vrije vetzuren in het plasma zijn verhoogd. In **hoofdstuk 3.1** wordt de hypothese beschreven dat in patiënten met hyperhomocysteinemie een toegenomen intracellulaire vorming van S-adenosylhomocysteine leidt tot een verlaagde intracellulaire concentratie van adenosine. Dit mechanisme vergroot de concentratiegradiënt van adenosine over de celmembraan, waardoor de gefaciliteerde diffusie van extracellulair adenosine via de ENT transporter de cel in toeneemt, hetgeen leidt tot een verminderde stimulatie van adenosinereceptoren. In **hoofdstuk 3.2** wordt beschreven hoe deze hypothese werd getoetst. We toonden aan dat in geïsoleerde erythrocyten homocysteine inderdaad de cellulaire opname van extracellulair adenosine versnelt en dat dit gepaard gaat met een toegenomen intracellulaire vorming van S-adenosylhomocysteine. Deze versnelde opname kon weer worden geremd met dipyridamol. Vervolgens bleek dat de adenosine-geïnduceerde vaatverwijding in de onderarm lager was in patiënten met een ernstige hyperhomocysteinemie op basis van een deficiëntie van cystathionine beta-synthase, en dat dit volledig kon worden hersteld door gelijktijdige toediening van dipyridamol. Deze effecten van homocysteine op de kinetiek van adenosine, die leiden tot een verminderde adenosinereceptorstimulatie, kunnen (mede) verantwoordelijk zijn voor het verhoogde cardiovasculaire risico van patiënten met hyperhomocysteinemie. **Hoofdstuk 3.3** geeft een beknopt overzicht van de verschillende studies die dit mechanisme hebben bestudeerd. In **hoofdstuk 3.4** toetsen wij de hypothese dat vrije vetzuren de extracellulaire adenosineconcentratie verhogen door remming van de adeninenucleotidetranslocator in de mitochondriële membraan (121). Via dit mechanisme zou endogeen adenosine betrokken kunnen zijn bij het ontstaan van de hyperdynamische circulatie die aanwezig is in patiënten met het metabool syndroom, die vaak een verhoogde concentratie vrije vetzuren in het plasma hebben. In gezonde vrijwilligers toonden we aan dat een acute stijging van de concentratie van vrije vetzuren in het plasma leidt tot vaatverwijding in de onderarm. Deze vaatverwijding bleek echter niet te berusten op een toegenomen stimulatie van adenosinereceptoren, aangezien coffeïne deze respons niet kon antagoneren. Deze resultaten suggereren dat adenosine geen rol speelt in de hemodynamische effecten van een verhoging van de vrije vetzuurconcentratie in het plasma, zoals in patiënten met het metabool syndroom.

Hoofdstuk 4 gaat over farmacologische beïnvloeding van het metabolisme en de receptorbinding van adenosine. In dit hoofdstuk richtten wij ons hoofdzakelijk op de mogelijkheid om met deze farmacologische interventies ischemie-reperfusieschade te beïnvloeden. Helaas ontbrak een eenvoudige methode om ischemie-reperfusieschade in de mens *in vivo* te bestuderen. Daarom hebben wij ^{99m}Tc-annexine A5 scintigrafie van de onderarm ontwikkeld om in deze behoefte te voorzien. Deze methode is gebaseerd op de binding van annexine A5 aan fosfatidylserinemoleculen, die al snel na het optreden van ischemie aan de buitenzijde van celmembranen verschijnen. Door recombinant annexine A5 te labelen met ^{99m}Technetium is het mogelijk deze vroege *marker* van ischemie-reperfusieschade zichtbaar te maken met een gammacamera. In **hoofdstuk 4.3** beschrijven we dat het intermitterend knijpen in een handkrachtmeter met de niet-dominante hand tijdens 10 minuten ischemie (geïnduceerd door het oppompen van een bloeddrukmanchet om de bovenarm tot 200 mmHg) leidt tot een significant hogere retentie van annexin A5 in de duimmuisspier van de experimentele hand ten opzichte van de controle hand ("targeting"). Vervolgens toonden we aan dat indien deze periode van ischemische inspanning wordt voorafgegaan door 10 minuten alleen maar ischemie van de onderarm, als een stimulus voor ischemische preconditionering, de annexinetargeting significant lager is. In de **hoofdstukken 4.1 en 4.2** wordt het fenomeen ischemische preconditionering en de mogelijkheid van farmacologische beïnvloeding van dit mechanisme uitgebreid beschreven. In **hoofdstuk 4.3** toonden wij ook aan dat de annexinetargeting na 10 minuten ischemische inspanning sterk kan worden verminderd door 20 minuten voorafgaand aan deze periode gedurende 10 minuten adenosine te infunderen in de arteria brachialis. ^{99m}Tc-annexine A5 scintigrafie blijkt dus een betrouwbare methode te zijn om in de mens *in vivo* ischemie-reperfusieschade te detecteren en tevens de beïnvloeding van deze schade door ischemische en farmacologische preconditionering. In patiëntenzorg wordt het gebruik van ischemische preconditionering sterk belemmerd doordat de stimulus voor preconditionering kort voor de schadelijke ischemische periode moet worden gegeven en het optreden van dit schadelijke ischemische insult meestal niet te voorspellen is. In **hoofdstuk 4.4** hebben we annexine A5 scintigrafie gebruikt om te onderzoeken of behandeling met dipyridamol een meer langdurige bescherming tegen ischemie-reperfusie kan bewerkstelligen. In gezonde vrijwilligers bleek de behandeling met dipyridamol (dipyridamol retard tabletten, 200 mg tweemaal per dag gedurende 1 week) in staat om de annexinetargeting in de duimmuisspier na 10 minuten ischemische inspanning significant te verlagen ten opzichte van onbehandelde proefpersonen. Omdat bekend is dat endogeen adenosine een belangrijke mediator is van het beschermende effect van ischemische preconditionering, veronderstelden wij vervolgens dat de adenosinereceptorantagonist coffeïne dit beschermende effect zou kunnen verminderen. In **hoofdstuk 4.5** wordt deze hypothese getoetst met het ^{99m}Tc-annexine A5 model. In een gerandomiseerde dubbelblinde studie in gezonde vrijwilligers toonden we aan dat coffeïne, in een plasmaconcentratie die in het dagelijks leven kan worden behaald door het drinken van 2 tot 4 koppen koffie, het beschermende effect van ischemische preconditionering volledig tenietdoet. Om te onderzoeken of deze bevindingen in de skeletspier kunnen worden geëxtrapoleerd naar de hartspier, hebben we een aanvullend model gebruikt om ischemie-reperfusieschade te meten in humane atriale trabekeltjes, die door de hartchirurg waren verwijderd tijdens een hartoperatie. In dit model werd herstel van contractiekracht na een periode van ischemie gebruikt als eindpunt van ischemie-reperfusieschade.

Ook in dit model bleek coffeïne het beschermende effect van ischemische preconditionering volledig te voorkomen.

Adenosine heeft niet alleen directe effecten op het cardiovasculaire systeem, maar heeft ook een sterk immunomodulerende werking: tijdens ontsteking neemt de adenosineconcentratie snel toe en stimulatie van adenosinereceptoren op cellen van het immuunsysteem remt vervolgens, als een negatief feedbackmechanisme, de ontstekingsrespons (10). Geneesmiddelen die de adenosineconcentratie verhogen, zouden dus ook een ontstekingsremmende werking kunnen hebben. Inderdaad is recent in verschillende diermodellen aangetoond dat de ontstekingsremmende werking van methotrexaat tot stand komt via adenosinereceptorstimulatie. Er zijn aanwijzingen dat methotrexaat door indirecte remming van de enzymen adenosine deaminase en AMPD respectievelijk de intracellulaire afbraak van adenosine remt en de extracellulaire vorming van adenosine verhoogt (584). Het doel van **hoofdstuk 4.6** was om dit effect van methotrexaat op het metabolisme van adenosine in de mens *in vivo* te bevestigen. In patiënten met gewrichtsontstekingen bleek de behandeling met methotrexaat gedurende 12 weken (15 mg per week) het vaatverwijdende effect van adenosine in de onderarm significant te versterken. Deze bevinding zou verklaard kunnen worden door verminderde intracellulaire afbraak van adenosine. Bovendien was het vaatverwijdende effect van dipyridamol significant hoger na behandeling met methotrexaat, hetgeen duidt op een hogere extracellulaire vorming van adenosine. Aangezien stimulatie van adenosinereceptoren vele gunstige cardiovasculaire effecten geeft, is het logisch om te veronderstellen dat methotrexaat ook potentieel gunstige effecten heeft op cardiovasculaire eindpunten. Recent bleek in een grote epidemiologische studie dat methotrexaat de cardiovasculaire mortaliteit in patiënten met reumatoïde arthritis verlaagt (607), hoewel een andere studie dit niet kon bevestigen (608). Interessant is ook de recente bevinding dat de behandeling met methotrexaat van patiënten met hartfalen de NYHA functionele klasse en de 6-minuten-looptest kan verbeteren (612). In **hoofdstuk 4.7** bespreken wij de mogelijkheid dat toegenomen adenosinereceptorstimulatie verantwoordelijk is voor deze effecten

In **hoofdstuk 4.8**, tenslotte, gebruiken wij dipyridamol als een farmacologisch instrument om de ENT transporter te remmen om te onderzoeken of endogeen adenosine een trigger is voor de "exercise-pressor" reflex. Deze reflex wordt gedefinieerd als de stijging van de arteriële bloeddruk die wordt veroorzaakt door inspanning van skeletspieren. Al vele eerdere studies hebben de hypothese trachten te toetsen dat deze reflex wordt geïnitieerd door activatie van afferente vezels van het sympathische zenuwstelsel in de skeletspier door adenosine, maar nog steeds is dit mechanisme niet opgehelderd. In hoofdstuk 4.8 lieten wij gezonde jonge proefpersonen intermitterend knijpen in een handkrachtmeter om de exercise-pressor reflex te activeren. Tegelijkertijd werd de adenosineconcentratie in het interstitium van de spier van de onderarm gemeten met behulp van microdialyse. Het bleek dat door het infunderen van dipyridamol in de arteria brachialis tijdens deze spierarbeid de stijging van de adenosineconcentratie werd versterkt, terwijl de exercise-pressor reflex niet werd versterkt. Deze bevindingen tonen overtuigend aan dat adenosine geen belangrijke rol heeft als trigger van de exercise-pressor reflex.

KLINISCHE IMPLICATIES EN TOEKOMSTPERSPECTIEVEN

Vele studies hebben aangetoond dat stimulatie van adenosinereceptoren de grootte van een myocardinfarct kan reduceren en tevens zijn er aanwijzingen dat adenosinereceptorstimulatie het ontstaan van atherosclerose kan remmen. Geneesmiddelen die de endogene adenosineconcentratie verhogen of direct adenosinereceptoren stimuleren zouden dus gunstig kunnen zijn voor patiënten met (een risico op) cardiovasculaire ziekten. Er zijn vele studies verricht in diermodellen naar de gunstige effecten van adenosine op cardiovasculaire eindpunten, maar er zijn slechts weinig humane *in vivo* onderzoeken gedaan. Twee studies in patiënten met een acuut myocardinfarct die behandeld werden met reperfusietherapie toonden aan dat intraveneuze infusie van adenosine gedurende 3 uur de grootte van het myocardinfarct, gemeten met ^{99m}Tc sestamibi tomografie, reduceert (511, 634). In deze studies was er echter geen significante verbetering van klinische eindpunten. De toepasbaarheid van adenosine in patiënten die een verhoogd risico hebben op cardiovasculaire incidenten wordt in de klinische praktijk bemoeilijkt door de extreem korte halfwaardetijd van adenosine, waardoor het nodig is een langdurig intraveneus infuus met adenosine te geven, en door de systemische bijwerkingen. Deze problemen zouden theoretisch omzeild kunnen worden door het gebruik van selectieve adenosine A_1- of A_{2A}-receptoragonisten. Er worden op dit moment al verschillende van deze agonisten onderzocht in fase III geneesmiddelenonderzoek, maar (nog) niet gericht op cardiovasculaire bescherming (65). Zo zijn selectieve adenosine A_1 receptoragonisten onderzocht om paroxysmale supraventriculaire tachycardieën te beëindigen (635) en selectieve adenosine A_{2A} receptoragonisten voor myocardscintigrafie (636).

Een andere, theoretisch zeer aantrekkelijke, manier om de beschermende effecten van adenosinereceptorstimulatie in de klinische praktijk te benutten, is het gebruik van *"allosteric enhancers"* van de adenosine receptor. Deze middelen versterken de gevoeligheid van de receptor voor de endogene ligand adenosine, waardoor de effecten van *allosteric enhancers* alleen optreden op die plaatsen waar op dat moment een hoge extracellulaire adenosineconcentratie is, zoals tijdens ischemie. Op deze manier is het mogelijk de effecten van adenosine op een bepaalde plaats in een orgaan en op een bepaald tijdstip selectief te versterken (637). *Allosteric enhancers* van de adenosine A_1 receptor zijn al getest in fase I geneesmiddelenonderzoek en fase II onderzoeken met deze middelen zijn reeds aangekondigd (T-62, King's Pharmaceuticals).

Ook geneesmiddelen die interfereren met het metabolisme of het transport van adenosine kunnen adenosinereceptorstimulatie bevorderen juist op die momenten en op die plaats waar dat nodig is. Dipyridamol verhoogt de endogene adenosineconcentratie, met name in situaties waarin de extracellulaire adenosinevorming is toegenomen, zoals tijdens ischemie. Dipyridamol heeft het voordeel dat het oraal toegediend kan worden, dat het weinig bijwerkingen heeft en dat het veilig is gebleken in grote klinische studies (558). In een van onze studies hebben we aangetoond dat behandeling met dipyridamol inderdaad ischemie-reperfusieschade in de onderarm, gemeten met annexine A5 scintigrafie, sterk kan verminderen. Deze bevindingen zijn goed verenigbaar met de resultaten van de recent gepubliceerde ESPRIT studie die was uitgevoerd in patiënten die een recent cerebrovasculair accident hadden doorgemaakt (638). Het bleek dat de combinatie van

dipyridamol en acetylsalicylzuur effectiever was dan alleen acetylsalicylzuur in de secundaire preventie van een myocardinfarct, een cerebrovasculair accident of vasculaire dood.

Acadesine (5-aminoimidazole-4-carboxamide riboside) is een ander geneesmiddel dat interfereert met het metabolisme van adenosine en op deze manier mogelijk de interstitiële adenosineconcentratie tijdens ischemie kan verhogen. In een recente studie werd onderzocht of acadesine (intraveneuze infusie gedurende 7 uur, vanaf 15 minuten voor start van de anesthesie) ischemie-reperfusieschade kon verminderen in patiënten die coronaire bypasschirurgie ondergingen. Het bleek dat acadesine niet de incidentie van een acuut myocardinfarct verminderde, maar wel de mortaliteit reduceerde in patiënten die een acuut myocardinfarct kregen, hetgeen past bij het fenomeen farmacologische preconditionering (639).

Helaas is de meest gebruikte farmacologisch actieve stof ter wereld, coffeïne, een adenosinereceptorantagonist. Theoretisch zou coffeïne dus een nadelig effect kunnen hebben in patiënten met hart- en vaatziekten. In dit proefschrift toonden wij aan dat coffeïne, in een plasmaconcentratie die in het dagelijks leven wordt gemeten na het drinken van enkele koppen koffie, het beschermende effect van ischemische precondionering volledig tenietdoet. Het is nog steeds controversieel of er een associatie bestaat tussen koffieconsumptie en de incidentie van hartinfarcten. Een recente epidemiologische studie toonde aan dat koffieconsumptie wel was geassocieerd met de incidentie van hartinfarcten, maar alleen in patiënten met een traag coffeïnemetabolisme, hetgeen suggereert dat coffeïne wel een rol speelt in deze associatie (579). In het licht van de beschermende werking van ischemische preconditionering op de grootte van een myocardinfarct, is het ook niet te verwachten dat coffeïne de incidentie van hartinfarcten vermindert, maar met name dat het een negatief effect heeft op de ernst van het myocardinfarct in patiënten met angina pectoris. Tevens is het te verwachten dat dipyridamol minder effectief is in patiënten die regelmatig koffie drinken.

Niet alleen de cardiovasculaire effecten van adenosine zouden gebruikt kunnen worden in de klinische praktijk, maar ook de sterke ontstekingsremmende effecten van adenosine en de effecten op het centrale zenuwstelsel, de nier en de long (recent uitgebreid beschreven in een review van Jacobson en Gua (65)). Het is aangetoond dat methotrexaat de extracellulaire adenosineconcentratie verhoogt en in diermodellen blijkt het anti-inflammatoire effect van methotrexaat zelfs volledig teniet te worden gedaan door adenosinereceptorantagonisten. Ook in mensen zijn er aanwijzingen dat de effectiviteit van methotrexaat in patiënten met reumatoïde arthritis lager is in patiënten die veel koffie drinken, hoewel een recente prospectieve cohortstudie deze associatie niet kon bevestigen (640). Gebaseerd op de bovengenoemde gegevens, denken wij dat het goed mogelijk is dat ook andere geneesmiddelen die de extracellulaire endogene adenosineconcentratie verhogen, zoals dipyridamol of acadesine, een anti-inflammatoir effect zouden kunnen hebben in patiënten met reumatoïde arthritis.

Geneesmiddelen die erop gericht zijn adenosinereceptorstimulatie te bevorderen zouden gebruikt kunnen worden in patiënten die een verhoogd risico hebben op het ontstaan van hart- en vaatziekten om de uitgebreidheid en misschien zelfs incidentie van vasculaire incidenten te

verminderen. De resultaten van de studies die in dit proefschrift worden beschreven, suggereren dat bepaalde groepen patiënten in het bijzonder zouden kunnen profiteren van deze geneesmiddelen. Het bleek bijvoorbeeld dat in patiënten met ernstige hyperhomocysteinemie de vasculaire effecten van adenosine verminderd zijn door een versnelde cellulaire opname van adenosine en dat dit volledig kon worden genormaliseerd door dipyridamol. Deze bevindingen suggereren dat in deze patiënten dipyridamol wellicht de cardiovasculaire mortaliteit zou kunnen verminderen.

Tenslotte kunnen polymorfismen of mutaties in genen die coderen voor eiwitten die betrokken zijn bij het metabolisme of de receptorbinding van adenosine een effect hebben op de werking van geneesmiddelen die tot doel hebben adenosinereceptorstimulatie te bevorderen. In dit proefschrift hebben we aangetoond dat het effect van dipyridamol op postocclusieve reactieve hyperemie beïnvloed wordt door het *AMPD1* genotype. Uit deze observaties volgt de hypothese dat de gunstige cardiovasculaire effecten van dipyridamol minder uitgesproken zijn in patiënten met de 34C>T variant van het *AMPD1* gen omdat de concentratiegradiënt van adenosine over de celmembraan, die bepalend is voor de gefaciliteerde diffusie van adenosine, in deze patiënten lager is. Genotypering kan in de toekomst niet alleen worden gebruikt om het effect van geneesmiddelen te voorspellen, maar kan ook worden gebruikt in de cardiovasculaire risicostratificatie van individuele patiënten. Wij hebben de hypothese dat, naast het 34C>T polymorfisme in het *AMPD1* gen, ook genetische variatie in andere eiwitten, zoals adenosine deaminase, adenosine kinase, de ENT transporter en specifieke adenosinereceptoren, een invloed kunnen hebben op het individuele cardiovasculaire risico.

Genotypes van eiwitten die betrokken zijn in het metabolisme en de receptorbinding van adenosine zouden dus gebruikt kunnen worden om het individuele cardiovasculaire risico van patiënten te voorspellen. Bovendien hebben dierexperimentele studies, *proof-of-concept* studies in gezonde vrijwilligers en enkele epidemiologische studies het aannemelijk gemaakt dat geneesmiddelen die de adenosinereceptorstimulatie beïnvloeden een beschermend effect kunnen hebben tegen hart- en vaatziekten. Om het gebruik van deze geneesmiddelen in de klinische praktijk te optimaliseren is meer basaal wetenschappelijk onderzoek om nieuwe stoffen te ontwikkelen die stimulatie van adenosinereceptoren kunnen beïnvloeden, is meer translationeel onderzoek nodig om de effecten van deze stoffen in de mens *in vivo* te bestuderen en is meer epidemiologisch onderzoek nodig om het effect van deze geneesmiddelen op harde cardiovasculaire eindpunten te onderzoeken in patiënten die een verhoogd risico hebben op hart- en vaatziekten.

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DANKWOORD

Het welslagen van translationeel wetenschappelijk onderzoek, waarbij beoogd wordt een theoretisch concept te onderzoeken in de mens *in vivo*, is per definitie volledig afhankelijk van de medewerking van proefpersonen. Meer dan tweehonderd gezonde vrijwilligers, veelal studenten, tien patiënten met gewrichtsontstekingen en tien patiënten met een zeldzame stofwisselingsziekte hebben als proefpersoon meegedaan aan de experimenten die in dit proefschrift worden beschreven. Zij verdienen in dit woord van dank zeker de eerste plaats.

Het verrichten van wetenschappelijk onderzoek zou ik nooit meer willen missen. De onbetwiste aanstichter hiervan is Paul Smits, bij wie ik in 1999 mijn eerste wetenschappelijke schreden heb gezet in een dierexperimentele studie naar het effect van stressgevoeligheid op vaatwandfunctie. Dat ik korte tijd later een congres in Milaan en een cursus in Glasgow mocht bijwonen, illustreert zijn immer slagvaardige optreden. Beste Paul, wat heb ik het getroffen dat ik toentertijd op jouw afdeling terecht ben gekomen. Op wetenschappelijk gebied ben je buitengewoon creatief, snel en accuraat. Maar niet minder belangrijk vind ik de prettige manier waarop je me begeleid hebt, je deur die letterlijk altijd open staat, je warme interesse in zaken die zich buiten de werkvloer afspelen (en je fraaie garderobe). Ik hoop van harte dat wij in de toekomst intensief met elkaar samen zullen blijven werken.

Gerard Rongen legde met het schrijven van de ZonMw aanvraag in 2002 het wetenschappelijk fundament voor dit proefschrift en verzorgde de dagelijkse begeleiding van de verschillende studies die in dit proefschrift staan beschreven. Gerard, dank je wel dat ik op jouw rijdende onderzoekstrein mocht springen en dat ik de verschillende studies met het door jou bedachte en gevalideerde "annexine model" mocht inkoppen. Ik bewonder je om je enorme wetenschappelijke vindingrijkheid, eigenwijsheid en doorzettingsvermogen. Het feit dat ik je meer dan eens 's ochtends vroeg achter de computer aantrof met je jas nog aan en het licht uit, illustreert je gedrevenheid. Ik spreek de hoop uit dat allerhande managementtaken je niet teveel zullen afhouden van datgene waar wat mij betreft je grootste kracht ligt: het bedenken en opzetten van innovatief en hoogstaand translationeel onderzoek. Ik vertrouw erop dat jouw "adenosine onderzoekslijn" een zeer succesvolle zal blijven en hoop dat ik nog veel met je mag samenwerken.

Petra van den Broek was de spil in het bedenken, opzetten en uitvoeren van alle *in vitro* studies die in dit proefschrift worden beschreven. Petra, dank je wel voor alle fraaie experimenten die je hebt uitgevoerd, de enorme berg werk die je hebt verzet voor onze genetische studies en de vruchtbare discussies. Nu ik "onze kamer" heb verlaten, zal ik je aanstekelijke lach zeker gaan missen. Ook Joke Verpalen wil ik danken voor haar inzet in het uitvoeren van de verschillende laboratoriumbepalingen.

De (ex-)collega-onderzoekers van de afdeling Farmacologie-Toxicologie en Interne Geneeskunde, Frans, Richard, Alexander, Bas, Patrick, Bart, Cees K, Cees T, Bastiaan, Henri, Egidia, Marlies, Marc en Peter dank ik graag voor de stimulerende en open sfeer die er altijd was en voor de leerzame discussies. Beste Alexander, ik geloof dat we beiden inmiddels feilloos het geluid van elkaars voetstappen herkennen, doordat we dagelijks wel even naar elkaars kamer liepen om bij te praten. Je oprechte interesse, je tolerante, beschouwende blik op de wereld, je zelfspot en je vrolijkheid met

enigszins tobberige ondertoon wekten vanaf het eerste moment grote sympathie. Ik ben erg blij met de vriendschap die hieruit is voortgekomen en ik vind het dan ook een eer dat je mijn paranimf wil zijn. Bart Ramakers heeft als student-assistent zijn grote organisatorische en sociale capaciteiten laten zien in het uitvoeren van de verschillende annexine studies. Bart, dank voor al je werk en zeker ook voor de gezellige avonden die we buiten het werk met elkaar hebben doorgebracht. Ik ken niemand die zo zelfverzekerd is als jij en daarbij toch zo sympathiek blijft. Ik vind het erg leuk dat ook jij recent een ZonMw AGIKO subsidie hebt gekregen om de mogelijke rol te onderzoeken van adenosine in pathogenese en behandeling van sepsis. Peter, ik hoop dat dit project het begin is van een vruchtbare samenwerking!

De opleiders van de Interne Geneeskunde in het UMC St Radboud, Jos van der Meer, Paul Stuyt en Jacqueline de Graaf wil ik danken voor hun flexibiliteit en steun, waardoor de combinatie van opleiding tot internist en promotieonderzoek buitengewoon soepel is verlopen.

Het merendeel van de klinische studies is uitgevoerd op de afdeling Klinische Fysiologie. Deze afdeling werd draaiende gehouden door Aarnout, Eugenie en Joost, die elk altijd klaar stonden als ik weer eens om een gunst vroeg. Bedankt hiervoor.

De meeste studies in dit proefschrift zijn tot stand gekomen door een intensieve samenwerking met onderzoekers van andere afdelingen uit het ziekenhuis. Enkelen hiervan wil ik met name noemen.

Wim Oyen van de afdeling nucleaire geneeskunde is vanaf het begin nauw betrokken geweest bij de ontwikkeling van de annexine scintigrafie. Wim, het kritisch doch snel lezen van mijn manuscripten leek vaak een nek-aan-nek race tussen Paul, Gerard en jou, maar jij was steevast de winnaar (record: binnen 2 uur gecorrigeerd manuscript terug). Dank voor de prettige samenwerking.

Barbara Franke van de afdeling Antropogenetica was onmisbaar voor de opzet en uitvoering van onze studies naar genetische determinanten van de cardiovasculaire effecten van adenosine. Barbara, bedankt voor alle zoektochten in de, voor mij ondoorgrondelijke, genetische databases, voor het opzetten van de verschillende bepalingen en voor het kritisch lezen van de manuscripten.

Henk Blom van het kinderneurologisch laboratorium wil ik graag danken voor de prettige samenwerking tussen zijn “homocysteine groep” en onze “adenosine groep”. Henk, ik vond onze gesprekken altijd erg prettig en leerzaam. Ik wens je veel succes met je nieuwe baan in Amsterdam. Frits Boers van de afdeling Interne Geneeskunde wil ik hartelijk danken voor zijn vertrouwen, dat bleek uit zijn bereidheid om mij “zijn patiënten” te laten benaderen met de vraag of ze zouden willen deelnemen aan mijn onderzoek.

Pilar Barrera van de afdeling Reumatologie was een spilfiguur in het bedenken en opzetten van de “methotrexaatstudie” en de rekrutering van de patiënten. Dank je wel hiervoor en veel sterkte de komende tijd.

Lieve ouders, jullie weten beiden goed hoe belangrijk een warm nest is voor later geluk. Jullie open en tolerante levensinstelling gaf me alle vrijheid om me te ontplooien. Jullie onvoorwaardelijke liefde en steun, grote interesse in alles wat ik doe en niet te stuiten enthousiasme over kunst en literatuur zijn onbetaalbaar. En wat fijn ook dat jullie de Universitaire Wereld van haver tot gort kennen, waardoor ik niets hoefde uit te leggen wanneer ik de vergelijking met “Het Bureau” weer eens maakte.

Lieve Onno, hoeveel kunnen broers van elkaar verschillen? Wij weten het antwoord. Ik ben heel blij dat jij mijn paranimf wil zijn. Wellicht het begin van een wat intensiever contact?

Lieve Gerri, zonder jou was dit proefschrift waarschijnlijk veel eerder klaar geweest. Jij hebt mij namelijk de ogen geopend voor vele andere facetten van het leven, die ik voor geen goud meer zou willen missen. Wat ben ik intens blij dat ik destijds voor mijn eerste coschap bij jou terecht kwam.

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CURRICULUM VITAE

Niels Riksen werd op 3 juli 1978 geboren in Arnhem en groeide op in Doorwerth. Na het behalen van zijn eindexamen aan het Stedelijk Gymnasium te Arnhem, werd hij in 1996 ingeloot voor de studie geneeskunde aan de Katholieke Universiteit Nijmegen (tegenwoordig Radboud Universiteit Nijmegen). Tijdens zijn studie, in 1999, begon hij met een onderzoek in ratten naar de invloed van stressgevoeligheid op endotheelfunctie op de afdeling Farmacologie-Toxicologie en sindsdien heeft hij die afdeling niet meer verlaten. In 2002 volgde hij de door de *European Society of Hypertension* georganiseerde *Hypertension Summerschool* in Glasgow.

In 2002 behaalde hij zijn artsexamen (cum laude) en begon hij aansluitend aan de opleiding tot internist. Hij ontving een ZonMw AGIKO stipendium (assistent geneeskundige in opleiding tot klinisch onderzoeker) om deze opleiding te combineren met promotieonderzoek op de afdeling Farmacologie-Toxicologie van het UMC St Radboud (Promotor: Prof. dr. P. Smits, Co-promotor: Dr. G.A. Rongen). De eerste 6 maanden van de opleiding tot internist werden doorgebracht in het Maxima Medisch Centrum te Veldhoven. Vervolgens startte hij met het onderzoek dat geleid heeft tot dit proefschrift, afgewisseld met enkele perioden opleiding tot internist in het UMC St Radboud te Nijmegen (Opleider: Prof. dr. J.W.M. van der Meer).

Tijdens zijn onderzoeksperiode won hij verscheidene prijzen voor beste mondelinge presentaties op congressen en tweemaal werd hem een van de Vasculaire Biologie Jaarprijzen toegekend voor het meest onderscheidende artikel op het gebied van de vasculaire biologie. Tenslotte kreeg hij in 2006 de Excellence^3 Award van de Novartis Foundation for Cardiovascular Excellence voor zijn wetenschappelijke en klinische prestaties. Hij woont samen met Gerri van den Broek en ze hopen in mei van dit jaar hun eerste kind te krijgen.